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EDITORIAL

Current issues in endoscope reprocessing and infection control during gastrointestinal endoscopy

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Abstract

The purpose of this article is to review the evidence regarding transmission of infection during gastrointestinal endoscopy, factors important in endoscope reprocessing and infection control, areas to focus on to improve compliance, and recent developments and advances in the field.

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Key words: Endoscopy; Infection; Disinfection; Reprocessing; Infection control

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INTRODUCTION

Gastrointestinal (GI) endoscopy is an important tool for the identification and treatment of disorders of the gastrointestinal tract. A thorough understanding of infection control and its application to GI endoscopy is crucial to prevent lapses in reprocessing and the possibility of transmission of infection. Endoscopes reprocessed appropriately, in accordance with reprocessing and infection-control guidelines, pose virtually no risk of transmission of patient-borne or environmental microorganisms. In the absence of defective equipment, every reported case of nosocomial infection associated with a contaminated GI endoscope has been linked to a specific breach or violation of at least one of several requisite reprocessing steps^[1].

TRANSMISSION OF INFECTION DURING GASTROINTESTINAL ENDOSCOPY

In a recent review of the published medical literature and the US FDA database, only 35 cases of transmission of infection during GI endoscopy have been reported in the last decade (again, all of which have been associated with breaches in reprocessing protocols)^[1] It has been estimated that 17 million lower GI procedures (colonoscopy and flexible sigmoidoscopy) are performed annually in the U.S.^[2], and a similar number of upper GI procedures are performed^[3,4]. If this procedure rate was constant during the past decade, these 35 cases occurred during an estimated 340 million procedures, yielding an infection rate that approaches 1 in ten million procedures. It is very likely that this simple calculation underestimates the actual incidence of transmission of infection, (unreported or unrecognized infections), however it does put the documented risk into perspective. Even if reported infections represented only 1% of the actual infection rate ("tip of the iceberg"), the transmission rate would be extremely low. We review the published literature with regard to documented cases of transmission of infection. Although there are few published data regarding some novel pathogens (SARS, Avian Influenza), their physical properties and anticipated susceptibility to current disinfection practices are discussed.

Viruses

Hepatitis C virus (HCV): There have been 8 cases of HCV transmission that have attributed to gastrointestinal endoscopy^[5-10]. A serious attempt at investigation (other than temporal association) and genotyping was performed in only three cases, in which transmission was firmly established by nucleotide sequencing^[7,8]. While both reports implicated inadequate disinfection of the colonoscope, they each also raised the possibility of contamination of syringes or multi-dose vials as the source of transmission. There have been several epidemiologic studies that have suggested an association between gastrointestinal endoscopy and HCV infection from several countries^[11-18]. The relevance of these observations may be limited. All of the studies relied on self-reports of exposure risks, which are unreliable for many of the socially stigmatized behaviors associated with hepatitis C virus (HCV) transmission, particularly IV substance abuse^[19]. A major concern for studies utilizing cross-sectional methodology is the inability to verify when infection occurred relative to

the procedure (i.e., the studies do not establish causality). It is not known whether currently accepted reprocessing protocols were being used during the periods under study, or even the degree to which endoscope reprocessing complied with these protocols. Finally, compliance with general infection control practices was not assessed, and the improper use (or reuse) of syringes and multiple-dose vials for sedation is increasingly recognized as an important risk factor for pathogen transmission^[20-23]. In a particularly notable example in 2001 it was extensively reported in the lay press that eight individuals having undergone endoscopy at a New York City endoscopy center had become infected with hepatitis C. Although initial reports suggested that it was due to the endoscopic equipment, a subsequent investigation by the New York City Department of Health concluded that the cause was in fact not the endoscopy, but rather improper handling of contaminated needles, syringes, and or multi-dose vials^[24]. Although transmission of infection resulted from a contact with the medical system, it did not reflect on the adequacy of endoscope reprocessing. Unless this aspect is controlled for in epidemiologic studies, the resulting association might reflect more on the need for better general infection control measures, rather than focusing on changes in endoscope reprocessing.

In fact, there are numerous studies demonstrating that HCV can be completely removed/eradicated from endoscopes during reprocessing^[25-29]. It could be argued that these results are somewhat artificial, and do not represent the state of endoscope reprocessing out in the community, i.e. endoscope reprocessing may have been more rigorous in the setting of the study. However, a landmark study comprising 8260 patients undergoing endoscopy who were tested for HCV seropositivity before and 6 mo after the procedure found no cases of seroconversion^[30]. This large study is the best evidence that appropriate endoscope reprocessing as performed in the community effectively prevents the transmission of hepatitis C virus.

Human immunodeficiency virus (HIV): While HIV may arguably be the most concerning pathogen for transmission of infection, it is actually a fragile virus that is highly susceptible to chemical disinfection. Mechanical cleaning alone can often completely remove the virus from contaminated endoscopes, and complete chemical disinfection is easily accomplished with glutaraldehyde^[26,31-34]. There are no reported cases of endoscopic transmission of HIV in the world literature.

Hepatitis B virus (HBV): There have been 5 cases of HBV transmission attributed to GI endoscopy^[6,35-38]. In the two cases reported by Morris and Birnie, reprocessing practices now known to be insufficient were used (failure to disinfect between patients, and when performed at the end of the day an inadequate disinfectant was used; failure to brush all channels in the second report)^[35,37]. The case mentioned in the introduction of the report as the impetus for the study by Seefeld in 1981 gives no details about the reprocessing practices and thus it is difficult to ascertain whether these were adequately performed. Reprocessing practices common at that time (now known to be suboptimal) included failure to disinfect between patients,

being performed only at the end of the day, the use of inadequate disinfectants, or inadequate exposure times^[39,40]. Fully immersible endoscopes were not introduced until 1983 (leaving a substantial part of the endoscope unexposed to the disinfectant) and the nature of the report suggests that the use of an aldehyde disinfectant was a new practice. In two later cases reported by Davis and Federman^[6,38], the association with endoscopy may be spurious. In each case, no investigation was performed to substantiate the association or evaluate other possible etiologies of transmission; in each case HBV infection was simply attributed to a prior colonoscopy due to the absence of self-reported risk factors. There are a number of prospective studies in which patients were followed for serologic evidence of HBV transmission following endoscopy. In six studies, a total of 223 patients in whom endoscopy was performed with an instrument known to have been used on a patient with HBV were followed for 6 mo; there were no seroconversions^[41-46]. Three other studies conducted in patient populations with relatively high rates of HBV infection followed a total of 600 seronegative patients for up to one year after endoscopy and found no episodes of seroconversion attributable to endoscopy^[47-49]. What makes these findings even more remarkable is that the reprocessing in all of the studies was, by current standards, suboptimal (endoscope "disinfection" performed with detergents rather than disinfectants, use of low-level disinfectants, or in one study exposure to glutaraldehyde for less than two minutes) implying that current reprocessing standards may provide an additional safety margin.

SARS-associated coronavirus (SARS-CoV): Severe acute respiratory syndrome is a recently identified, potentially fatal atypical pneumonia clinically characterized by fever, cough, myalgias, and shortness of breath. It was first recognized in 2003 in the Guangdong Province of China, but has since affected more than 8000 people in 25 countries across 5 continents. The etiologic agent has been identified as the SARS-associate coronavirus (SARS-CoV), a virus not previously endemic to humans. The mechanism of transmission is predominantly infectious respiratory droplets, although aerosolization and fomites may also be contributory^[50]. SARS CoV has been identified in respiratory secretions and feces, and can be found in intestinal epithelial cells of affected individuals, suggesting that GI endoscopes can be exposed to potentially infectious material^[51,52]. It is not known whether the virus can be transmitted *via* the oral route. One recent study evaluated a variety of hand and surface disinfectants (low-level disinfectants), as well as a glutaraldehyde-based medical instrument disinfectant. The study found that SARS-CoV was readily inactivated by all the disinfectants^[53]. Thus it appears likely that current reprocessing protocols and high-level disinfectants are adequate to prevent transmission of the virus as a result of endoscopy itself; there are no published reports of transmission of SARS *via* contaminated GI endoscopes. However, the endoscopy suite itself may serve as a vector of transmission (including the health care workers in it), and the most important factors in preventing nosocomial transmission are respiratory precautions (face masks) and appropriate hand hygiene^[54].

Avian influenza A virus (H5N1): The H5N1 virus is a specific subtype of the avian influenza A virus that has recently received significant media attention. This virus infects the respiratory and gastrointestinal tracts of poultry, is highly contagious, spreads rapidly, and has been identified as the cause of several recent outbreaks of avian influenza in a number of countries. Of greatest concern to public health, this avian influenza virus can mutate quickly and cross the species barrier. Once thought to be exclusive to birds, the H5N1 virus was first reported to infect humans in Hong Kong in 1997^[55]. Since then, more cases of human infection with this highly pathogenic strain have been reported in Asia. These cases resulted from direct or close contact with infected poultry, infected respiratory tract secretions and feces, and/or contaminated environmental surfaces. Person-to-person transmission of the H5N1 influenza virus has also been reported but to date this mode of transmission appears to be very limited. The recent surge of the H5N1 (avian influenza) virus has raised questions about whether current endoscope disinfection protocols are sufficient in the healthcare setting to prevent its transmission. Although there are few data specific to GI endoscopy, there are several reasons to suggest that current reprocessing guidelines are sufficient. Because the natural reservoir hosts of the virus are waterfowl, the risk of nosocomial patient-to-patient transmission during any type of surgical/endoscopic procedure is (currently) negligible. Geographically, there have been no reported cases in North America. More importantly, the H5N1 virus is lipid-enveloped, and like other viruses in this class (e.g. other influenza viruses, SARS virus, HIV), they are the easiest to inactivate by physical or chemical decontamination methods compared to all other types of pathogenic microorganisms. An EPA-registered cleaner/disinfectant labeled to achieve low-level (or intermediate-level) disinfection is sufficient to remove and destroy virtually all enveloped viruses including the H5N1 virus. Therefore, current recommended reprocessing practices for GI endoscopes and other types of flexible endoscopes—specifically, cleaning, followed by at least high-level disinfection and drying—provide a sufficient margin of safety to prevent GI endoscopes from transmitting the H5N1 virus from patient-to-patient. Additionally, cleaning followed by either low-level disinfection (which lacks a tuberculocidal claim) or intermediate-level disinfection (which includes a tuberculocidal claim, but not a sporicidal claim) of environmental surfaces (e.g., bedside tables, bed stands, table tops) are similarly sufficient to destroy the H5N1 virus and prevent its nosocomial spread. Review of the cleaner/disinfectant's label is necessary to ensure proper dilution and adequate contact time to effect its outcome. Frequent hand washing is also necessary to prevent transmission of the H5N1 virus.

Bacterial infections

Salmonella: Since 1974, there have been 48 cases of endoscopic transmission of various *Salmonella* species^[56-64]. Each of these cases has been associated with at least one breach in currently accepted reprocessing guidelines, usually a failure to mechanically clean the internal instrument channel, although the use of an inappropriate

disinfectant, or an inadequate disinfection time were also common. It is interesting to note that there have been no reported cases of salmonella transmission since the publication in 1988 of standardized cleaning and disinfection recommendations from the American Society of Gastroenterology (ASGE), the Society for Gastrointestinal Nurses and Associates (SGNA), and the British Society of Gastroenterology (BSG)^[65,66].

Pseudomonas aeruginosa: *Pseudomonas aeruginosa*, a Gram-negative bacillus that is an opportunistic pathogen found widely in the environment, is of particular concern in the endoscopy setting due to its predilection for a moist environment (e.g., endoscope water/irrigation systems, wet internal channels after reprocessing, or even the hospital water supply itself). Unlike *salmonella*, which does not appear to be a persistent infection control problem, *Pseudomonas aeruginosa* continues to pose a challenge to endoscope reprocessing, and is the most commonly reported organism responsible for transmission of infection during endoscopy. There have been 216 reported cases of *Pseudomonas aeruginosa* transmission^[67-83]. While early reports of pseudomonas infection resulting from endoscopy were most commonly related to inadequate cleaning or the use of inadequate disinfectants, later reports tend to implicate three major areas: (1) the automated endoscope reprocessor (AER) or the water supply to the endoscope that become colonized with the organism, (2) failure to disinfect the elevator channel of duodenoscopes, and most importantly, (3) failure to completely dry any or all channels of the endoscope with a 70% alcohol solution and forced air.

H pylori: There have been 12 reported cases of *H pylori* infection that have been attributed to endoscopic transmission^[84-88]. In each case, suboptimal cleaning and disinfection were implicated (most commonly an inappropriate liquid chemical germicide, or LCG). Several studies have addressed whether current reprocessing protocols are sufficient to eradicate the organism. Three studies reported the presence of the organism after cleaning and disinfection (2 using culture methods and the third using PCR amplification)^[89-91]; however in two of the studies an inadequate exposure time to the LCG was used, and in the third the reprocessing protocol was not described at all. In a study using both manual and automated disinfection, well-described conventional reprocessing protocols consistent with currently accepted guidelines resulted in 100% eradication of the organism as determined by PCR analysis^[92]. Of interest, there have been numerous studies suggesting a higher incidence of *H pylori* in healthcare workers, and in most studies particularly endoscopy-related staff, than in age-matched controls^[93-104]. Four studies, however, found no such association^[105-108], although it has been suggested that failure to find an association may be due to inadequate study power or residual confounding from other exposure risks. The possibility of transmission of pathogens from patients to health care workers underscores the need for good general infection control practices, including the use of appropriate personal protective equipment (PPE), such as gloves, gowns, masks and protective eyewear.

Miscellaneous Organisms: One study raised the

possibility of 2 cases of endoscopic transmission of *Tropheryma whipplei*, the organism responsible for Whipple's disease, although this conclusion is tenuous at best. The sole rationale for this association was that each patient had undergone an endoscopy for upper gastrointestinal tract symptoms approximately 3 years preceding the final diagnosis^[109]. Given the rarity and novelty of the disease, it would not be difficult to determine if any other cases had been reported at the institution, and whether or not they had undergone endoscopy prior to either index case, thus providing at least a temporal association. The authors themselves make the point that the organism has a very slow growth rate, and a more plausible explanation is that the initial procedure was performed for symptom investigation in patients that already harbored the organism in an early stage of the disease that was simply undetected. There are also methodologic problems that undermine the conclusions of the article about the *in-vitro* resistance of the organism to high-level disinfection^[110]. Two studies have reported transmission of various *Enterobacteriaceae* strains during biliary endoscopy (ERCP), including *E coli*, *Klebsiella*, *Enterobacter*, and *Serratia marcescens*^[82,111]. In both studies, flaws in the cleaning and disinfection process were noted.

Transmissible spongiform encephalopathy (TSE)

Creutzfeldt-Jakob disease (CJD) and variant CJD (vCJD) are degenerative neurological disorders known as transmissible spongiform encephalopathies (TSEs). These disorders are associated with aberrant proteins referred to as prions. The incubation period from acquisition to overt clinical disease is thought to range from months to years (in some cases decades). Prions present a unique challenge to endoscope reprocessing because of their extraordinary resistance to traditional disinfection and sterilization processes. Because endoscopic procedures such as percutaneous endoscopic gastrostomies (PEGs) are commonly performed on patients with dementia that may harbor occult TSEs, concern had been expressed that endoscopy may serve as a vector for transmission of these agents.

Creutzfeldt-Jakob disease (CJD): CJD is the most common form of TSE, and approximately 90% of CJD is sporadic^[112,113]. Iatrogenic transmission has been reported after use of contaminated neurosurgical equipment, or after inadvertent inoculation with infectious materials (dura matter grafts, pituitary hormones). From animal studies, transmission is most efficient with intracerebral or intracranial inoculation; peripheral (extracranial inoculation) requires substantially larger doses. Unfortunately, there is general agreement that currently available liquid chemical germicides used for high level disinfection of gastrointestinal endoscopes are unable to eliminate prion infectivity. Although one study claimed to show that peracetic acid could inactivate prion protein^[114], the controls (without peracetic acid) also demonstrated the absence of detectable protein after two cycles. Other studies have demonstrated that peracetic acid is unable to reliably inactivate prions^[115-118]. While oral transmission has been reported in primates, it requires ingestion of large

quantities of highly infectious material^[119]. Although prions have recently been found in the spleen, skeletal muscle, and olfactory epithelium of individuals with sporadic CJD^[120,121], fortunately, they are not found in bodily secretions or gut mucosa^[122,123]. Because gastrointestinal endoscopes do not contact prion-containing tissue or secretions, and even trace contamination would be reduced or eliminated with simple mechanical cleaning, thus rendering any potential inoculum far below the threshold for oral acquisition, infection control experts have stated that currently accepted cleaning and disinfection protocols should be adequate for reprocessing endoscopes^[113,124,125]. The adequacy of current guidelines for endoscope reprocessing with regard to CJD are supported by the fact that there have been no reported cases in the world literature of transmission of CJD or other TSEs by endoscopy.

Variant Creutzfeldt-Jakob disease (vCJD): Variant Creutzfeldt-Jakob disease (vCJD) is a more recently described TSE that is believed to be caused by the consumption of contaminated beef products containing the bovine spongiform encephalopathy (BSE) agent^[126]. The disease may also require a person to have a susceptible genotype. To date approximately 155 cases of vCJD have been reported in the world. The one reported case in the United States was found in a 22 year-old patient that had contracted the disease in the UK and developed symptoms after moving to the US^[127]. Unlike CJD, the prions associated with vCJD can be detected in the lymphoid tissue of affected individuals (although at much lower concentrations than the CNS), notably the tonsil, the appendix, and possibly the ileum and rectum (with obvious relevance to GI endoscopy)^[126,128-131]. However, this tissue was approximately 50% less infective than CNS tissue when homogenated and injected intracerebrally in mice^[132]. The infectivity of intact tissue that might be encountered at endoscopy and subsequent transmissibility is unknown, but would presumably be much lower. Given the virtual absence of this disease in the US, rigorous adherence to current guidelines for the cleaning and disinfection of endoscopes would seem to be the best protection for the public. There is no evidence that changes to current endoscopic practices or endoscope reprocessing guidelines are warranted, but these should be responsive to new information as it evolves. The fact that the risk of transmission of prions associated with CJD or vCJD is negligible should not be seen as advocating complacency. In the absence of an endoscope-compatible germicide that can completely and reliably inactivate prion infectivity, there is a window of vulnerability (albeit a small one) and further work is needed. Conversely, we should not rush to adopt a new technology without considering the overall risk to the patient. As an example, the adoption of single-use surgical equipment for tonsillectomy in the UK (to prevent the theoretical risk of prion transmission) actually led to a substantial increase in the rate of postoperative hemorrhage, and subsequently the recommendation was abandoned by health authorities in the UK^[133-135].

ENDOSCOPE CLEANING AND DISINFECTION

The US Multi-Society guidelines and the WGO-OMGE/OMED guidelines provide comprehensive recommendations for reprocessing gastrointestinal endoscopes^[136,137]. Briefly, endoscope-reprocessing is a three-stage process that includes: (1) pre-processing, or cleaning the endoscope and its detachable components using a detergent solution and brushes; (2) processing, or high-level disinfection of the endoscope using an LCG (in the US, cleared by the Food and Drug Administration [FDA]) followed by thorough water rinsing to remove residual LCG from the instrument; and (3) post-processing, which includes proper handling and storage of the endoscope. This third and final step also includes drying the endoscope and its internal channels after terminal water rinsing^[138]. Review of these guidelines is beyond the scope of the present article, but the importance of endoscope drying and water quality will be discussed below.

In addition to patient-to-patient transmission of pathogens during endoscopy, environment-to-patient transmission of gram-negative bacteria during GI endoscopy has been reported, primarily during endoscopic retrograde cholangiopancreatography (ERCP)^[76,79,81,82]. Due in part to the anatomy, physiology, and sterile nature of the biliary tract, the design of the side-viewing duodenoscope (elevators channel), and the nature and characteristics of the procedure, ERCP is probably more vulnerable to bacterial infection than other GI endoscopic procedures. Several of these reports highlight, first, an association between wet or improperly dried GI endoscopes and true and pseudo-outbreaks of waterborne microorganisms, and, second, the abrupt termination of these outbreaks following the implementation of endoscope drying. Moreover, some of these reports identified the rinse water used during endoscope reprocessing as the source of the microorganisms responsible for these outbreaks^[76,79,81,82]. Despite the thoroughness and success of cleaning and high-level disinfection, failure to dry the endoscope can render the reprocessing procedure invalid and clinical use of the endoscope an infection risk. The contribution of post-processing in general and drying in particular to the prevention of disease transmission *via* a GI endoscope cannot be overstated.

Endoscope drying and storage

Although the importance of cleaning and high-level disinfection of GI endoscopes after each procedure is well recognized, the contribution of post-processing—particularly endoscope drying and storage—to the prevention of disease transmission and nosocomial infection is sometimes overlooked. Surveys indicate that not all GI endoscopy units dry the endoscope after water rinsing and prior to reuse or storage^[139,140]. Rinse water that is not removed during drying and remains in the endoscope's narrow internal channels between patient procedures or more importantly during storage can provide the ideal environment for waterborne microorganisms to colonize and multiply. Indeed, cases of nosocomial infection due to the transmission of

microorganisms that have colonized and proliferated in the moist internal channels of inadequately dried and improperly stored endoscopes to the patients undergoing GI endoscopy have been reported, resulting in patient injury and death^[70,71,76,79,82].

Allen^[76] reported that bile cultures from ten patients who had undergone ERCP were contaminated with *Pseudomonas aeruginosa*. Inadequate drying after reprocessing of the ERCP endoscope using an AER was identified as the cause of this outbreak. *P. aeruginosa* was found to have survived and proliferated in the moist internal channels of an ERCP endoscope that transmitted this bacterium to patients during ERCP. Contamination ended and this outbreak was terminated only after modifying the facility's reprocessing procedure to include after cleaning and high-level disinfection a manual drying step achieved by suctioning alcohol through the ERCP endoscope's channels followed by air drying. Classen *et al.* reported the identification of seven cases of *P. aeruginosa* bacteremia within 5 d following ERCP^[79]. In addition to each infected patient having undergone the first or only scheduled ERCP of the day, the mean duration between reprocessing the ERCP endoscope and its clinical use was significantly longer for infected patients than for matched controls, suggesting that improper storage of the endoscope played a role in this outbreak. Each patient was found to be infected with the same serotype of *P. aeruginosa* as microbiologically sampled from, among other surfaces, the tap water basin used to rinse the ERCP endoscopes with water after disinfection. This report's authors suggest that inadequate drying of the endoscope's channels prior to storage was, at least in part, responsible for this outbreak. *P. aeruginosa* remaining in the endoscope's moist internal channels after reprocessing and water rinsing likely colonized and multiplied to high numbers during overnight storage, posing an increased risk of nosocomial infection to the first scheduled patient of the day. Among other control measures including more frequent changing of, and addition of chlorine into, the tap water bath, no additional infections were identified once the endoscope's channels were flushed with 70% alcohol followed by forced air.

Provided the endoscope is properly reprocessed and dried prior to storage, reprocessing the endoscope immediately before its first use of the day does not appear to be necessary^[141]. There are few data that provide insight into the number of days a specific type of GI endoscope may remain in storage without posing an infection risk and requiring reprocessing before its reuse. Two studies, however, suggest that properly reprocessed and dried endoscopes may remain in storage for five to seven days without requiring reprocessing before reuse^[142,143]. The type of endoscope, its frequency of reuse, and the effectiveness of the reprocessing and drying protocols may all be factors that influence and affect the number of days a GI endoscope can remain safely in storage without posing a risk of bacterial colonization and nosocomial infection. Research to determine storage intervals is encouraged.

Water quality

While proper mechanical cleaning (stage 1) and high-level disinfection (stage 2) are crucial to the prevention

of disease transmission during GI endoscopy, the success of an endoscope reprocessing procedure also depends on the adequacy of the drying step and the microbial quality of the water used to remove the residual liquid chemical germicide from the endoscope after disinfection. In general, three types of water are used to rinse endoscopes after chemical immersion: tap water, bacteria-free water, and "sterile" or "sterile filtered" water. Despite their label claim, however, all of these water types, including "sterile" water, have been linked to bacterial contamination and nosocomial infection following endoscopy^[76,79,81,82,144-147]. Because the water used to rinse the endoscope after chemical immersion is not generally microbiologically monitored (i.e., periodically cultured), its microbial quality is almost always unknown^[148]. The rinse water contacts the endoscope after high-level disinfection (or "liquid sterilization"), whether achieved manually or using an automated endoscope reprocessor (AER), and in many cases just prior to clinical use (if not exposed to a drying cycle). Thus any contamination of the rinse water will inevitably lead to contamination of the endoscope regardless of the potency, strength, or effectiveness of the preceding cleaning process or of the LCG, AER, or automated processing system. Drying the endoscope during post-processing (stage 3) is necessary, no matter the claimed microbial quality of the rinse water, to prevent potential re-contamination of the endoscope with waterborne microorganisms during terminal water rinsing^[149].

The importance of microbiological monitoring of the rinse water used during the reprocessing of GI endoscopes is controversial. Whereas some countries encourage this practice^[150,151], others (including the United States) do not, having concluded that the relationship between the presence of bacteria in the rinse water and nosocomial infection has not been adequately defined^[148]. As discussed previously, contamination of endoscopes with waterborne, gram-negative bacteria has been linked to adverse patient outcomes. Unless the rinse water is monitored to evaluate its microbial quality and content, the potential exists for the rinse water to contain pathogenic microorganisms capable of re-contaminating the endoscope during terminal water rinsing, compromising the effectiveness of the reprocessing procedure, invalidating the disinfection (or "liquid sterilization") claim, and posing a risk of nosocomial infection. Periodic sampling of the rinse water used during endoscope reprocessing also provides independent verification that the bacterial filter, which is used to improve the microbial quality of the rinse water used by virtually all AERs, is working properly and producing "bacteria-free" or "sterile" water as labeled^[148]. Bacterial filters have a limited life-span and have been reported to fail, allowing bacteria to pass, resulting in true and pseudo outbreaks^[82,151-154]. It is for these and other reasons that some reports recommend microbiological monitoring of the rinse water, to preempt re-contamination of the endoscope^[148,150,151]. The importance and necessity of this practice, and the recommendation that the rinse water be bacteria-free or sterile^[151,155], is minimized, however, by thoroughly drying the endoscope after completion of every reprocessing cycle (i.e., between-

patient procedures and before storage) to prevent the transmission of waterborne microorganisms that may reside in the rinse water^[81]. Professional organizations and governmental agencies are encouraged to develop standards that establish permissible levels of waterborne bacteria (and endotoxins) for the rinse water, to ensure its microbial quality does not pose an infection risk during endoscopy.

GENERAL INFECTION CONTROL

When discussing infection control during gastrointestinal endoscopy, attention is almost invariably focused on the adequacy of the endoscope reprocessing for the prevention of patient-to-patient transmission of pathogens. Good general infection control practices are critical for the prevention of infection in any medical setting. There are now numerous examples of pathogen transmission from the improper use/reuse of syringes, multiple-dose drug vials, and IV equipment^[21-23,156-159]. As mentioned previously, the widely publicized outbreak of HCV at an endoscopy clinic in New York that was initially attributed to improper endoscope reprocessing was subsequently found to be due to contaminated multiple-dose sedative medication vials^[24].

As alluded to earlier in the discussion regarding the higher rate of *H pylori* infection in healthcare workers, patients may serve as a vector for transmission of infection to endoscopy staff. Although rare, there are case reports of transmission of HCV from a blood splash to the conjunctiva of health care workers^[160-162], and one case of bacterial conjunctivitis from a splash during colonoscopy^[163]. One study reported that the overall splash rate to the eyes was 4.1%, and was not altered by the use of video endoscopy, highlighting the need for appropriate personal protective equipment^[164]. Compliance with published infection control guidelines is necessary to minimize the potential for nosocomial transmission of infection, both to patients and health care workers.

NEW TECHNOLOGIES

Cleaning, disinfection and sterilization processes and technologies are crucial to the prevention of disease transmission and nosocomial infection caused by both patient-borne and environmental microorganisms and other types of contagions. While current reprocessing products are effective and meet the requirements of infection-control and endoscope-reprocessing guidelines, the search for better-performing LCGs that are more effective in less time without damaging gastrointestinal endoscopes continues. The market's demand for new and more innovative products remains strong, primarily because the advantages that current reprocessing products offer are typically offset by disadvantages that limit their applications and usefulness. As an example, although 2% glutaraldehyde is cost-effective, can be used during manual reprocessing or with most automated endoscope reprocessors, and has a long track record of effectiveness and endoscope compatibility, its vapors may be irritating to endoscopy staff and requires a

relatively longer exposure time. Alternatively, ortho-phthalaldehyde (OPA) is tuberculocidal in 5 minutes, does not require activation, and has not been reported to cause endoscope damage. But these favorable characteristics are overshadowed to some extent by OPA's propensity for staining instrument surfaces and skin, significantly higher cost, and contraindication for reprocessing urological equipment, due to the identification of serious allergic reactions in some bladder cancer patients who underwent repeated cystoscopies. Development of an LCG that is compatible with flexible endoscopes and other delicate materials, rapidly tuberculocidal (and sporicidal), cost-effective, environmentally-friendly, not associated with allergic reactions for patients or healthcare workers, does not contain a high concentration of soapy surfactants or require heating to achieve high level disinfection, and can be used during both manual and automated reprocessing has proved challenging. While current products may satisfy several of these criteria, none satisfies all of them.

In the quest to improve the current standard of reprocessing for GI endoscopes and other types of flexible endoscopes, several new products have been developed and recently cleared by the US *Food and Drug Administration* (FDA) for marketing in the U.S. These new products and technologies range from enzymatic detergents that claim to facilitate the removal of different types of organic debris including fats from the surfaces of flexible endoscopes; to glutaraldehyde-based disinfecting solutions that are rapidly tuberculocidal at room temperature; to low-temperature sterilization processes that use a hydrogen peroxide based-plasma labeled to achieve sterilization of bronchoscopes and other instruments with narrow lumens or channels. Sheath-based technologies that cover the flexible endoscope's insertion tube to prevent its contact with the patient and contamination have been applied to GI endoscopes and other types of flexible endoscopes with measured results. Whether any of these reprocessing products or sheathed technologies improves the status quo and provides clear advantages over currently available technology will need to be established. Some of these new reprocessing technologies are discussed, below.

Cidex OPA concentrate (Advanced Sterilization Products, or ASP; FDA clearance No. K032959)

This liquid chemical concentrate was cleared by the FDA in April, 2005, and is manufactured by ASP. It contains 5.75% (w/v) *ortho*-phthalaldehyde (OPA) and is a concentrated form of its predecessor, Cidex OPA (0.55% *ortho*-phthalaldehyde), which was cleared by the FDA in October, 1999. This concentrate is mixed with tap water to achieve a diluted, single-use solution of 0.05% OPA, which is labeled to achieve high-level disinfection of flexible endoscopes and other types of reusable medical and dental devices in 5 min at an elevated temperature of 50°C. Cidex OPA Concentrate is contraindicated for manual reprocessing and is labeled exclusively for use in the EvoTech Integrated Endoscope Disinfection System, an automated endoscope reprocessor also recently cleared by the FDA and marketed by ASP.

Because this product will be marketed in tandem with the Evotech disinfecting system, the success of Cidex

OPA Concentrate will depend on the success of the Evotech, and vice versa. Whether healthcare facilities will replace 2% glutaraldehyde or Cidex OPA-both of which are versatile and can be used manually or with any automated endoscope reprocessor-with Cidex OPA Concentrate and its accompanying reprocessor, is unclear. Because its active ingredient is *ortho*-phthalaldehyde, which has not been reported to cause endoscope damage, Cidex OPA Concentrate is likely to be compatible with a wide range of delicate and heat-sensitive instruments and, therefore, is not likely to void the warranty provided with most endoscope models. As with any LCG, adequate room ventilation is required.

Of particular clinical importance, Cidex OPA Concentrate, like Cidex OPA, is likely to be contraindicated for reprocessing cystoscopes and other types of urological instrumentation to be used to treat patients who have a history of bladder cancer, due to reports of an association between these patients experiencing anaphylaxis-like reactions after having undergone repeated cystoscopies and contact with cystoscopes reprocessed using Cidex OPA (*ortho*-phthalaldehyde). Whether contraindications for Cidex OPA Concentrate will include cystoscopes and possibly other types of flexible endoscopes is unclear. The labeling of Cidex OPA Concentrate does not include a sporicidal claim.

Aldahol III high level disinfectant (Healthpoint, LTD; FDA clearance No: K041360)

This product was cleared by the FDA in May, 2005, and uses a novel mixture of two well known chemicals to achieve high-level disinfection of flexible endoscopes. Aldahol III (pH 7.6 after activation) contains a mixture of 3.4% glutaraldehyde and 26% isopropanol and, like most 2% glutaraldehyde formulations, can be reused for up to 14 d, depending on several factors including usage and how effectively the instrument is cleaned and dried prior to chemical immersion. This product achieves high-level disinfection in 10 min at 20°C (room temperature), suggesting that the addition of alcohol (i.e., 26% isopropanol) to a solution of 3.4% glutaraldehyde enhances its tuberculocidal properties, reducing the time and temperature required to achieve high-level disinfection. Aldahol III is sporicidal in 10 h at 20°C, and its concentration of glutaraldehyde is monitored for effectiveness using chemical test strips, to ensure its concentration is above 2.1%, this product's minimum effective concentration.

Aldahol III contains isopropyl alcohol, which is ordinarily flammable and can, under certain conditions, pose a risk of injury to staff and to patients if not adequately removed from the endoscope during thorough water rinsing. Whether the chemistry of this product eliminates this risk is unclear. Prolonged immersion of the endoscope in solutions that contain isopropyl alcohol (as opposed to brief flushing of the endoscope's internal channels with alcohol to facilitate drying) may damage the endoscope. Most published reprocessing guidelines contraindicate the use of LCG that contain high concentrations of surfactants. Before using this product to reprocess flexible endoscopes in an automated endoscope

reprocessor (AER), contact both respective manufacturers to ensure use of Aldahol III will not void the endoscope's or AER's warranty. Also, the powdered contents of the activator added to each gallon of this solution may not immediately dissolve into solution as required prior to its use.

Accide™ high level disinfectant and sterilant (Minntech; FDA clearance No: K041984)

This product, which is similar in chemical composition to some products that are used for dialyzer reprocessing (Actril, Renalin) and endoscope reprocessing (Peract 20), was cleared by the FDA in May, 2005. It contains a mixture of 8.3% hydrogen peroxide and 7.0% peracetic acid. These two chemical agents, referred to as Solution 1 and Solution 2, are packaged separately and for reasons of chemical instability are mixed by medical staff at the time of use. Accide is labeled to achieve high-level disinfection of flexible endoscopes and other types of reusable medical and dental devices in 5 min at 25°C. Accide is sporicidal in 5 h at 25°C and can be reused for a maximum of 5 d, depending on several factors including usage (i.e., the number of endoscopes reprocessed using the solution) and how effectively the instrument is cleaned and dried prior to chemical immersion. This product's concentration of peracetic acid is monitored for effectiveness using chemical test strips, to ensure its concentration is above 1900 parts-per-million, which is Accide's minimum effective concentration.

Accide can be reused for a maximum of 5 d. As a result, its cost-per-cycle is likely to be more expensive than other LCGs (such as 2% glutaraldehyde) which may be reused for as many as 14 d. Accide uses a mixture of hydrogen peroxide and peracetic acid which may be incompatible with delicate materials and result in endoscope damage. Contact the endoscope's manufacturer before using Accide to ensure its use will not void the endoscope's warranty. Like other recently introduced LCGs that require an elevated immersion temperature to be effective, accide may be contraindicated for manual reprocessing and require use of an automated endoscope reprocessor (AER)

STERRAD NX system (Advanced Sterilization Products, or ASP; FDA clearance No. K042116)

This sterilizing system was cleared by the FDA in April, 2005, and launched in the U.S. in May, 2005. It uses an electrical field in a low-temperature, negative-pressure chamber to convert a solution of hydrogen peroxide and water to a hydrogen peroxide plasma cloud that contains ultraviolet light and free radicals with sporicidal properties. Known as the Sterrad NX, this device is marketed as a safe and rapid-acting sterilizer designed to replace ethylene oxide (EtO) gas sterilizers, which pose a potential hazard to personnel and the environment and require as long as 24 h, with aeration, to complete a processing cycle. This device is labeled to sterilize a wide range of surgical instruments, including flexible endoscopes, although it is indicated only for those models of flexible endoscopes that feature a single working channel (no air, water, or accessory channels) with an inner diameter of at least 1

mm and a length not longer than 850 mm. Importantly, the Sterrad NX is contraindicated for reprocessing gastrointestinal endoscopes.

In summary, transmission of infection during gastrointestinal endoscopy is an extremely rare event, and in each case has been associated with a breach in currently accepted reprocessing guidelines or faulty equipment. When appropriate reprocessing guidelines are followed, endoscopes pose virtually no risk of transmission of infection. It is also important that general infection control measures, particularly during the administration of intravenous sedative agents, be meticulously adhered to in the endoscopy suite. Although novel pathogens may pose particular challenges to endoscope disinfection, current protocols appear to be sufficient to protect the patient from cross-infection. However, endoscope design improvements and new germicides to facilitate reprocessing and specifically address these challenges should be encouraged.

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GASTRIC CANCER

Study of Sonic hedgehog signaling pathway related molecules in gastric carcinoma

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Abstract

AIM: To study the expression of Sonic hedgehog pathway-related molecules, Sonic hedgehog (Shh) and Gli1 in gastric carcinoma.

METHODS: Expression of Shh in 56 gastric specimens including non-cancerous gastric tissues, gastric adenocarcinoma, gastric squamous cell carcinoma was detected by RT-PCR, *in situ* hybridization and immunohistochemistry. Expression of Gli1 was observed by *in situ* hybridization.

RESULTS: The positive rate of Shh and Gli1 expression was 0.0%, 0.0% in non-cancerous gastric tissues while it was 66.7%, 57.8% respectively in gastric adenocarcinoma, and 100%, 100% respectively in gastric squamous cell carcinoma. There was a significant difference between the non-cancerous gastric tissues and gastric carcinoma ($P < 0.05$). Elevated expression of Shh and Gli1 in gastric tubular adenocarcinoma was associated with poorly differentiated tumors while the expression was absent in gastric mucinous adenocarcinoma.

CONCLUSION: The elevated expression of Shh and Gli1 in gastric adenocarcinoma and gastric squamous cell carcinoma shows the involvement of activated Shh signaling in the cellular proliferation of gastric carcinogenesis. It suggests Shh signaling gene may be a new and good target gene for gastric tumor diagnosis and therapy.

INTRODUCTION

Sonic hedgehog (Shh), a mammalian homologue of *Drosophila* secreted morphogen Hedgehog, is crucial for the development of various embryonic tissues in invertebrate and vertebrate development, including brain, spinal cord, axial skeleton, limbs, lungs, gut, and hematopoietic cells^[1-4]. Shh is synthesized in epithelial cells. Its membrane receptor Patched1 (Ptc1) is expressed in adjacent mesenchymal cells. Ptc1, a 12-transmembrane protein, does not transduce the intracellular signals by itself. This task is executed by smoothed (Smo), a 7-transmembrane protein that belongs to heterotrimeric G protein-coupled receptor (GPCR) family. In the absence of Shh, Ptc1 suppresses the activity of Smo by binding to Smo. Upon Shh stimulation, Shh binds to Ptc1, and then Smo is de-repressed from the suppression of Ptc1 and is able to transduce the intracellular signals to transcription factor Gli. Gli transfers the signals into the nucleus^[5].

Shh is implicated in the early expansion of developing midbrain and also in the proliferation of granular cell precursors in the cerebellum^[6-8]. In human, likewise in experimental models, the activated Shh signaling is thought to predispose to the development of tumors^[9-12]. The role of the Shh pathway in the regulation of oncogenic transformation is a new and exciting field. The study by Berman *et al* suggests that this pathway may well offer the potential for new treatments for medulloblastoma^[11]. The recent finding that Shh pathway activity is important for growth of small cell lung cancers, a tumor type not associated with Gorlin's syndrome, suggested that other, non-Gorlin's tumors might require Shh pathway activity for growth^[12-14]. In the longer term, better understanding of the regulatory role of this pathway may offer new targets for therapeutic manipulation.

Gastric cancer is the second most common cause of cancer-related death in the world. Many Asian countries, including Korea, China, and Japan, have very high rates of gastric cancer^[15]. By far, the mechanism of Hedgehog signal in gastric cancer is still unclear. Here we studied the role of Shh signaling pathway-related molecules, Shh and Gli1, in gastric adenocarcinoma and squamous cell carcinoma by RT-PCR, immunohistochemistry and *in situ* hybridization.

MATERIALS AND METHODS

Samples

A total of 56 specimens of gastric tissues were used in our study. Fifty-six patients, who had undergone curative gastrectomy between 2002 and 2005 from the Department of General Surgery, Shandong Qilu Hospital affiliated to Shandong University, or from Jinan Central Hospital, Shandong Province, China, were enrolled in this study. All of the resected primary tumors were histologically examined by hematoxylin and eosin staining, and confirmed by pathologists. These samples included five resection specimens of non-cancerous gastric tissues, ten well differentiated tubular adenocarcinoma, ten moderately differentiated tubular adenocarcinoma, fifteen poorly differentiated adenocarcinoma, seven papillary adenocarcinoma, three gastric mucinous adenocarcinoma and six gastric squamous cell carcinoma.

Immunohistochemistry

Representative formalin fixed and paraffin embedded tissue sections (6 μ m thick) were used for immunohistochemistry with specific antibodies to human Shh (Cat# 9024, Santa Cruz Biotechnology Inc, Santa Cruz, CA). First, tissue sections were deparaffinized, followed by rehydration with serially decreased concentrations of ethanol, and immersed in 3% H₂O₂ (in distilled water) for 10 min to inhibit endogenous peroxidase activity. Following antigen retrieval in citrate buffer (pH 6.0), the tissue sections were incubated with normal goat serum to block nonspecific antibody binding for 20 min at room temperature. The sections were then incubated with primary antibodies (at 1:200 dilution) at 37°C in humid chambers for 2 h. After washing with PBS 3 times, the sections were incubated with biotinylated secondary antibody (goat anti-rabbit IgG) and streptavidin conjugated to horseradish peroxidase for 20 min at 37°C, followed by PBS wash. The sections were incubated with DAB substrate for less than 30 min. Haematoxylin was used for counterstaining. Negative controls were performed in all cases by omitting the first antibodies.

RT-PCR

Total RNA of cells was extracted by an using RNA extraction kit (Promega). Total RNA templates were isolated from 10 μ g sections (10 μ m) of paraffin-embedded tissue as described elsewhere^[16]. Three micrograms of total RNA were reverse transcribed by using M-MLV reverse transcriptase (Promega) with mixture of oligo(dT)₁₅ and random primers (Promega). One-tenth of each RT

reaction mixture was then subjected to PCR amplification using Taq DNA polymerase (TAKARA). The PCR primers for detecting specific transcripts are as below: for *Shh* sense 5'-ACCGAGGGCTGGGACGAAGA-3' and antisense 5'-ATTTGGCCGCCACCGAGTT-3' respectively. Following denaturation at 94°C for 10 min, 35 PCR cycles were performed at 94°C for 60 s, at 52°C for 50 s, and at 72°C for 60 s. The PCR products were analyzed by 0.7% agarose gel electrophoresis.

In situ hybridization

Shh (L38518) was subcloned in PbluescriptKS⁺. The linearized pBlueScript-shh was obtained by digestion with *Sph* I or *Xmn* I restriction endonuclease. Gli1 (X07384) was cloned into pBluescript M13+KS. The plasmid was digested with *Nru* I to generate the sense fragment, with *Nde* I to generate the antisense fragment. Sense and antisense probes were obtained by T3 and T7 *in vitro* transcription using a kit from Roche (Mannheim, Germany). Tissue sections (6 μ m thick) were mounted onto Poly-L-Lysine slides. Following deparaffinization, tissue sections were rehydrated in a series of dilutions of ethanol. To enhance signal and facilitate probe penetration, sections were immersed in 0.3% Triton X-100 solution for 15 min at room temperature and in proteinase K (2 mg/mL) solution for 20 min at 37°C, respectively. The sections were then incubated with 4% (v/v) paraformaldehyde/PBS for 5 min at 4°C. After washing with PBS and 10 \times saline citrate, the slides were incubated with prehybridization solution (50% formamide, 50% 4 \times SSC) for 2 h at 37°C. The probe was added to each tissue section at a concentration of 1 g/mL and hybridized overnight at 42°C. After high stringency washing (2 \times SSC twice, 1 \times standard saline citrate twice, 0.5 \times SSC twice at 52°C), sections were incubated with an alkaline phosphatase-conjugated sheep anti-digoxigenin antibody, which catalyzed a color reaction with the NBT/BCIP (nitro-blue-tetrazolium/5-bromo-4-chloro-3-indolyl phosphate) substrate (Roche, Mannheim, Germany). Blue indicated strong hybridization. As negative controls, sense probes were used in all hybridization and no positive signal was observed.

Statistical analysis

Analysis was performed by using chi-square test and correlation analysis with SPSS 11.0 software. $P < 0.05$ was considered statistically significant.

RESULTS

Expression of Shh-mRNA in non-cancerous gastric tissues and gastric carcinoma

Total RNA extracted in paraffin-embedded tissue was pure enough to meet the need for RT-PCR. The length of RT-PCR product was 211 bp. The results of RT-PCR showed that expression of Shh was different in various tissue, including non-cancerous gastric tissues, well differentiated and poorly differentiated adenocarcinoma. Expression of Shh was higher in gastric adenocarcinoma than in non-cancerous gastric tissues (Figure 1), which was confirmed by *in situ* hybridization. The results of *in situ* hybridization

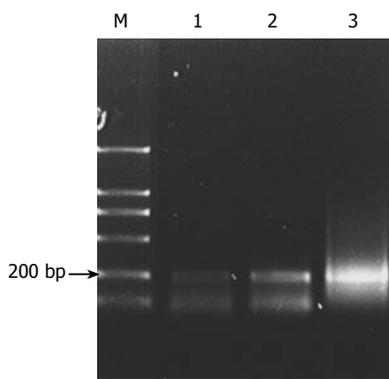


Figure 1 Results of Shh RT-PCR. M: MarkerDL-2000; 1: Non-cancerous gastric tissues; 2: Well differentiated tubular adenocarcinoma; 3: Poorly differentiated tubular adenocarcinoma.

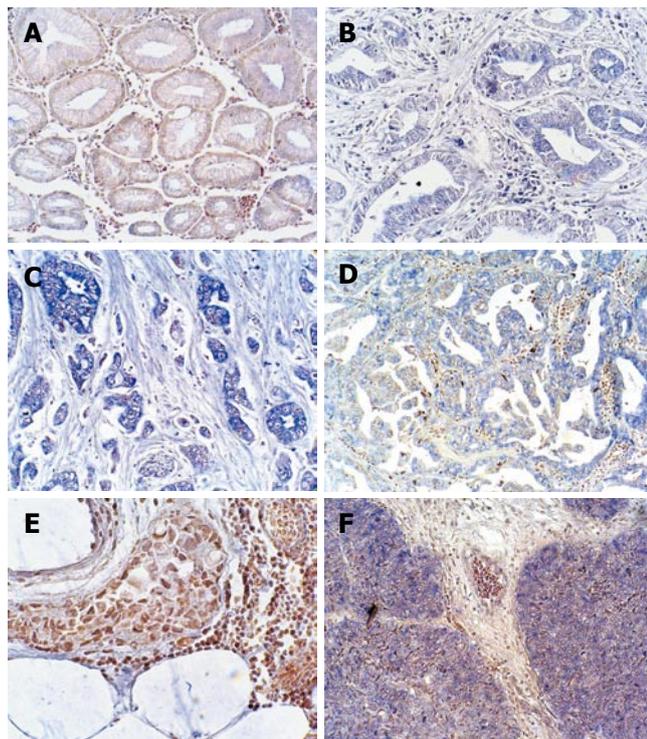


Figure 2 Results of Shh expression by *in situ* hybridization, blue represents positive. A: Non-cancerous gastric tissues ($\times 100$); B: Well-moderately differentiated tubular adenocarcinoma ($\times 100$); C: Poorly differentiated tubular adenocarcinoma ($\times 100$); D: Papillary adenocarcinoma ($\times 100$); E: Mucinous adenocarcinoma ($\times 200$); F: Squamous cell carcinoma ($\times 100$).

showed that Shh mRNA was expressed in cytoplasm of the fundic glandular epithelium and some stromal cells. Shh was not or lowly expressed in non-cancerous gastric tissues' glandular epithelium while overexpressed in about 66.7% (30/45) adenocarcinoma patients and in 100% (6/6) gastric squamous cell carcinoma (Figure 2A-F) (Table 1). In tubular and papillary adenocarcinoma glandular epithelium, the expression of Shh showed an increasing trend among well differentiated, moderately differentiated and poorly differentiated adenocarcinomas (Figure 2B-D), while expression of Shh was absent in gastric mucinous adenocarcinoma (Figure 2E). In gastric squamous cell carcinoma, expression of Shh was obvious (Figure 2F). There was a significant difference between the non-cancerous gastric tissues and gastric carcinoma ($P < 0.05$) (Table 1).

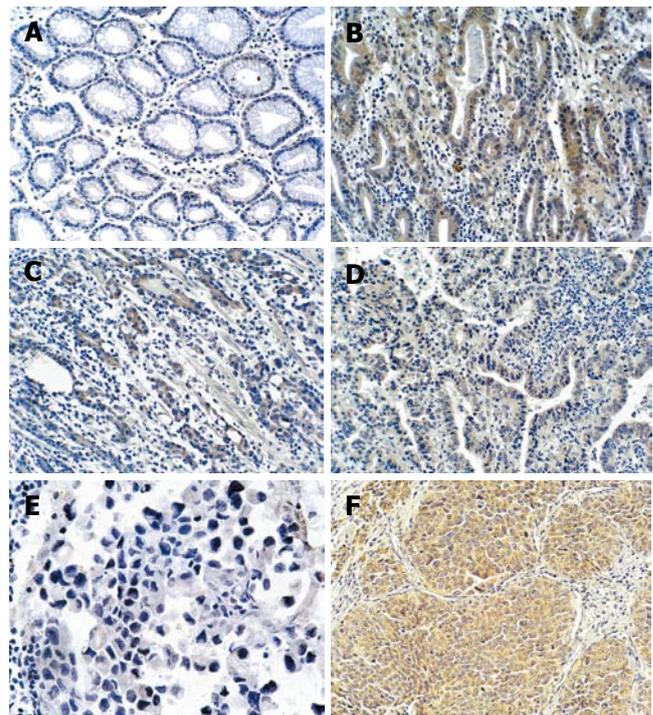


Figure 3 Results of Shh expression by immunohistochemistry (brown represents positive). A: Non-cancerous gastric tissues ($\times 100$); B: Well-moderately differentiated tubular adenocarcinoma ($\times 100$); C: Poorly differentiated adenocarcinoma ($\times 100$); D: Papillary adenocarcinoma ($\times 100$); E: Mucinous adenocarcinoma ($\times 200$); F: Squamous cell carcinoma ($\times 100$).

High expression level of Shh protein in gastric carcinoma

The results of immunohistochemistry showed that Shh staining was detected in the fundic glandular epithelium and in the stroma of the stomach (Figure 3A-F). Shh protein was not or lowly expressed in non-cancerous gastric tissues (Figure 3A) and was highly expressed in gastric adenocarcinoma and gastric squamous cell carcinoma (Figure 3B-D, F). The results of Shh expression in various gastric tissue were consistent with results of *in situ* hybridization. Shh was overexpressed in gastric adenocarcinoma epithelium and squamous cell carcinoma epithelium compared with non-cancerous stomach tissues glandular epithelium. Shh expression was increased with malignant degree aggravation in gastric adenocarcinoma (Figure 3B-D), while there was no or less expression of Shh in gastric mucinous adenocarcinoma (Figure 3E).

Expression of transcription Gli1 in non-cancerous gastric tissues and gastric carcinoma

As an Shh signaling transcription, Gli1, was detected by *in situ* hybridization. It showed Gli1 was not or lowly expressed in non-cancerous gastric tissues glands and highly expressed in epithelium cytoplasm, in 57.8% (26/45) gastric adenocarcinoma and 100% (6/6) squamous cell carcinoma (Figure 4A-F) (Table 1). There was a higher expression level of Gli1 in squamous cell carcinoma (Figure 4F). Also, it presented an increasing trend in tubular adenocarcinoma with malignant degree aggravation (Figure 4B-D), while there was no or lower expression of Shh in gastric mucinous adenocarcinoma (Figure 4E). There was a significant difference between the non-cancerous gastric

Table 1 Summary of expression of Shh and Gli1 in various types of gastric tissues

	Tumor (n)	Expression of Shh n (%)	Expression of Gli1 n (%)	P value
Non-cancerous gastric tissues	5	0 (0.0)	0 (0.0)	0.01 < P < 0.05
Adenocarcinoma	45	30 (66.7)	26 (57.8)	
Tubular				
Well differentiated (WD)	10	4 (40.0)	3 (30.0)	
Moderately differentiated (MD)	10	8 (80.0)	7 (70.0)	
Poorly differentiated (PD)	15	13 (86.7)	12 (80.0)	
Papillary	7	5 (71.4)	4 (57.1)	
Mucinous	3	0 (0.0)	0 (0.0)	
Squamous cell carcinoma	6	6 (100)	6 (100)	

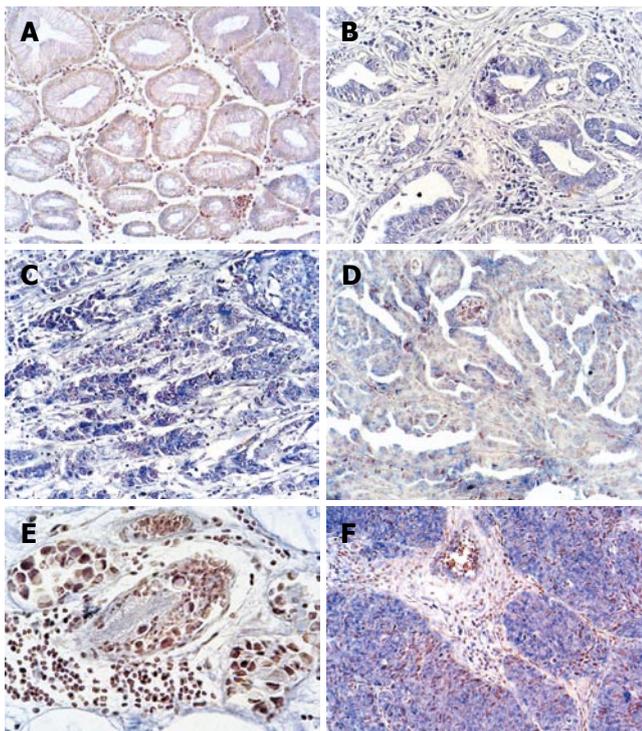


Figure 4 Results of Gli1 by *in situ* hybridization (blue represents positive). **A:** Non-cancerous stomach tissues ($\times 100$); **B:** Moderately differentiated tubular adenocarcinoma ($\times 100$); **C:** Poorly differentiated tubular adenocarcinoma ($\times 100$); **D:** Papillary adenocarcinoma ($\times 100$); **E:** Mucinous adenocarcinoma ($\times 200$); **F:** Squamous cell carcinoma ($\times 100$).

tissues and gastric carcinoma ($P < 0.05$) (Table 1).

DISCUSSION

Shh is an important endodermal signal in endodermal-mesenchymal interaction during vertebrate development of the gut tube, especially to gastric gland^[17]. Because Shh is expressed consistently in the endodermal epithelium of the gut throughout the period of organogenesis and late embryonic life, it is natural to suppose that its expression is crucial for the differentiation and maintenance of gut tube epithelium. Indeed, the heterozygous mutant mouse for Shh shows various gastrointestinal defects, such as intestinal transformation of stomach, annular pancreas, and duodenal stenosis^[18]. Some of these defects may be caused by the absence of Shh effects on the mesenchyme, while others may reflect a direct action of Shh on epithelial

cells. Although the expression of Shh is essential for the development of gut tube and the fundic glands of the adult gastrointestinal tract, the role of Shh expression has not been described in the gastric carcinogenesis^[14,15]. We examined Sonic hedgehog signaling pathway related molecules expression in non-cancerous gastric tissues and in various types of gastric carcinoma. The results indicated that Shh protein expression is closely correlated with glandular epithelium differentiation in adenocarcinoma and gastric squamous cell carcinoma. The increase of Shh expression in tumor tissue is consistent with the results of Berman *et al*^[14].

Gastric cancer is one of the leading causes of cancer death worldwide. Because of its heterogeneity, gastric cancer has been an interesting model for studying carcinogenesis and tumorigenesis^[15]. This study mainly detected the expression of Shh and Gli1 in various types of gastric carcinoma. The elevated expression of Shh and Gli1 in gastric adenocarcinoma and squamous cell carcinoma suggested the involvement of activated Shh signaling in the cellular proliferation of certain type of gastric carcinoma. Interestingly, there was no activation of Shh signaling in mucinous adenocarcinoma. It implies that there may exist different mechanisms in various gastric carcinoma.

In all the tumors with elevated levels of Gli1, expression of Shh was also increased, suggesting that overexpression of Shh may be responsible for elevated expression of Gli1 in gastric adenocarcinoma. However, a high level of sonic hedgehog expression was not always accompanied by elevated Gli1 expression in adenocarcinoma (Table 1), indicating additional regulatory mechanisms for the hedgehog pathway activation. In addition to the hedgehog overexpression, other genetic alterations may be required to activate the hedgehog pathway in gastric cancers, such as Wnt, Notch signal pathway. Further study of the relationship between Sonic Hedgehog and other signal pathways may provide more evidences for a new and good target gene for gastric cancer diagnosis and therapy.

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GASTRIC CANCER

Correlative studies on uPA mRNA and uPAR mRNA expression with vascular endothelial growth factor, microvessel density, progression and survival time of patients with gastric cancer

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Abstract

AIM: To investigate the correlations between the expression of urokinase-type plasminogen activator (uPA) mRNA, uPA receptor (uPAR) mRNA and vascular endothelial growth factor (VEGF) protein and clinicopathologic features, microvessel density (MVD) and survival time.

METHODS: *In situ* hybridization and immuno-histochemistry techniques were used to study the expressions of uPA mRNA, uPAR mRNA, VEGF and CD34 protein in 105 gastric carcinoma specimens.

RESULTS: Expressions of uPA mRNA, uPAR mRNA and VEGF protein were observed in 61 (58.1%) cases, 70 (66.7%) cases and 67 (63.8%) cases, respectively. The uPA mRNA and uPAR mRNA positive expression rates in infiltrating-type cases (73.7%, 75.4%), stage III-IV (72.1%, 75.4%), vessel invasion (63.2%, 69.9%), lymphatic metastasis (67.1%, 74.4%) and distant metastasis (88.1%, 85.7%) were significantly higher than those of the expanding-type ($\chi^2 = 15.57, P = 0.001; \chi^2 = 6.91, P = 0.046$), stage I-II ($\chi^2 = 19.22, P = 0.001; \chi^2 = 16.75, P = 0.001$), non-vessel invasion ($\chi^2 = 11.92, P = 0.006; \chi^2 = 14.15, P = 0.002$), non-lymphatic metastasis ($\chi^2 = 28.41, P = 0.001; \chi^2 = 22.5, P = 0.005$) and non-distant metastasis ($\chi^2 = 12.32, P = 0.004; \chi^2 = 17.42, P = 0.002; \chi^2 = 11.25, P = 0.012; \chi^2 = 18.12, P = 0.002$). The VEGF positive expression rates in infiltrating-type cases (75.4%), stage III-IV (88.5%), vessel invasion (82.9%), lymphatic metastasis (84.3%) and distant metastasis (95.2%) were significantly higher than those of the expanding-type ($\chi^2 = 9.61, P = 0.021$),

stage I-II ($\chi^2 = 16.66, P = 0.001$), non-vessel invasion ($\chi^2 = 29.38, P = 0.001$), non-lymphatic metastasis ($\chi^2 = 18.68, P = 0.005$), and non-distant metastasis ($\chi^2 = 22.72, P = 0.007; \chi^2 = 21.62, P = 0.004$). The mean MVD in the specimens positive for the uPA mRNA, uPAR mRNA and VEGF protein was markedly higher than those with negative expression groups. Moreover, a positive relation between MVD and uPA mRNA ($rs = 0.199, P = 0.042$), uPAR mRNA ($rs = 0.278, P = 0.035$), and VEGF ($rs = 0.398, P = 0.048$) expressions was observed. The mean survival time in cases with positive uPA mRNA, uPAR mRNA and VEGF protein expression or MVD value ≥ 54.9 was significantly shorter than those in cases with negative expression or MVD value < 54.9 .

CONCLUSION: uPA and uPAR expressions are correlated with enhanced VEGF-induced tumor angiogenesis and may play a role in invasion and nodal metastasis of gastric carcinoma, thereby serving as prognostic markers of gastric cancer.

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Key words: Stomach neoplasm; Urokinase-type plasminogen activator; Urokinase-type plasminogen activator receptor; Vascular endothelial growth factor; Prognosis

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INTRODUCTION

Degradation of extracellular matrix (ECM) and basement membrane is essential for tumor invasion and metastasis. The ECM is degraded by extracellular proteolytic enzymes, such as metalloproteases and serine proteases. Plasminogen activators (PA) catalyze the conversion of the inactive pro-enzyme plasminogen to plasmin^[1]. Plasmin acts to degrade the ECM and activates latent enzyme, such as type-IV collagenase. Among the plasminogen activators, urokinase-type plasminogen activator (uPA) and uPA receptor (uPAR)

have been reported to play an important role in tumor progression. uPA activation occurs on the cell surface after binding to its specific receptor and is regulated by the number of uPAR^[2]. The relevance of uPA or uPAR in tumor progression has been demonstrated by the poor prognosis of patient with a high content of uPA or uPAR in tumor tissue.

Angiogenesis is important for tumor growth and metastasis^[3,4]. The formation of tumor microvessels is stimulated by angiogenic factors, especially vascular endothelial growth factor (VEGF). Previous studies have shown that over-expression of VEGF is correlated with the progression of human malignancies, and a significant correlation has been demonstrated between microvessel density (MVD) and VEGF expression in tumor cells^[5,6]. Recently, a role of the PA system in tumor angiogenesis has also been demonstrated^[7]. Simultaneous study of uPA, uPAR and VEGF is of practical value to reveal the mechanism and progression of gastric carcinoma. In this study, we observed the expressions of uPA, uPAR and VEGF in gastric carcinomas so as to explore the relationship of the carcinoma angiogenesis with its infiltration, metastasis and other carcinoma biological behaviors as well as prognosis.

MATERIALS AND METHODS

Patients and tumor tissues

One hundred and five gastric carcinoma samples were collected in our hospital from October 1986 to November 1998. All the samples were fixed with formaldehyde and embedded in paraffin. Complete over 5 years follow-up data were available for all these cases (follow-up ended in October 2002). Survival period was calculated from the day of operation to the end of the follow-up or to the date of death due to recurrence and metastasis. In all samples, uPA and uPAR mRNA expressions were detected using *in situ* hybridization, and VEGF and CD34 expressions were detected using immunohistochemistry. The average age of the cases was 57.6 (range 38-78) years and the male to female ratio was 2:1. According to the standard classification of WHO (1999), 17 cases had papillary adenocarcinomas, while 37, 34, 9 and 8 had the tubular adenocarcinomas, poorly differentiated adenocarcinomas, mucinous adenocarcinomas, and signet-ring cell carcinomas, respectively. Highly and intermediately differentiated carcinomas were found in 63 cases, while poorly and undifferentiated carcinomas were found in 42 cases. Forty-eight cases showed expansive growth, while 57 cases had infiltrative growth of carcinomas; and 20, 24, 32 and 29 cases had T1, T2, T3, and T4 carcinoma, respectively. Carcinomas with vascular invasion were found in 76 cases, and non-vascular invasion carcinomas in 29 cases. Seventy cases had lymph node metastasis, while 35 cases had no lymph node metastasis. Distant metastasis of carcinomas were found in 42 cases (liver metastasis: 18 cases, peritoneum metastasis: 24 cases), while no distant metastasis in 63 cases. Twenty control samples were collected from the same gastric mucosa 5 cm away from the carcinoma tissues.

Histological treatment

In order to avoid the RNase contamination, all the glass slides, slide covers and stain containers were treated with 100 g/L DEPC for 24 h. Gloves were used when handling tissue cutting and 100 g/L SDS was used to clean the cutter. All the sections were spread on glass using 100 g/L DEPC-treated ddH₂O. The tissues were cut into 5-7 μ m thickness and kept in 4°C, and foil covered for HE stain, immunohistochemistry and *in situ* hybridization.

Reagents

Digoxin-labeled oligonucleotides probes of uPA (No. MK1194) and uPAR (No. MK2117) were purchased from Boshide Biological Technology Limited Company, Wuhan, China. The sequences were 5'-CTAGGCCTGGGGAAACACAATTACTGCAGG-3' and 5'-TGTCTACACGAGGGTCTCACACTTCCTGGA-3' for uPA, and 5'-GCCTCTTGGGCAGTGCATGCAGTGT-3', 5'-GAGCTGTGAGAGGGCCCGCAGCAAAGCCT-3' and 5'-ACTGCCGTGACCAATGAATCAATGTCTGG-3' for uPAR. Mouse anti-human VEGF and mouse anti-human CD34 and SP kit were purchased from Zhongshan Biotech Co., Beijing, China. The working concentrations of VEGF and CD34 were 1:100 and 1:150, respectively.

In situ hybridization

All the slides, cover-slips and other containers were autoclaved and treated with 100 g/L DEPC-treated ddH₂O for 24 h. All the buffers were also treated with 100 g/L DEPC. The tissues were routinely treated before *in situ* hybridization. DEPC (100 g/L)-treated ddH₂O was used to spread out the sections. Moderate temperature was used for drying the sections; gradient ethanol was used for dehydration with 30 mL/L H₂O₂ incubation for 10 min at room temperature. Digestion was enabled with pepsin at 37°C for 20 min, followed by washing thrice with 0.5 mol/L PBS (5 min each time). Then 20 mL hybridization solution was used for each group with probes, sealed, incubated in wet chamber for 20 h at 45°C. Then the slides were washed with 2 \times SSC for 5 min, followed by incubation with 20 mL hybridization stabilization solution (2 mL A solution, 18 mL B solution) at 45°C for 5 h in a wet chamber. Post-hybridization washing was done with 2 \times SSC-0.05 \times SSC for 2 h, and then the slides were blocked with normal serum at 37°C for 30 min. After directly adding mouse-anti-digoxin antibody for 1 h at 37°C, slides were washed thrice with 0.5 mol/L PBS (5 min each time), followed by incubation with streptavidin-biotin complex (SABC) at 37°C for 20 min and biotin-peroxidase at 37°C for 20 min. Finally, the slides were washed thrice with 0.5 mol/L PBS (5 min each time), stained with DAB for 10 min and counterstained with hematoxylin solution for 8 min. Hybridization solution and RNase-treated sample served as negative controls.

Immunohistochemistry

Consecutive 5-7 μ m paraffin-embedded tissue sections were subjected to immunostaining according to the streptavidin peroxidase (SP) methods. Briefly, the tissue

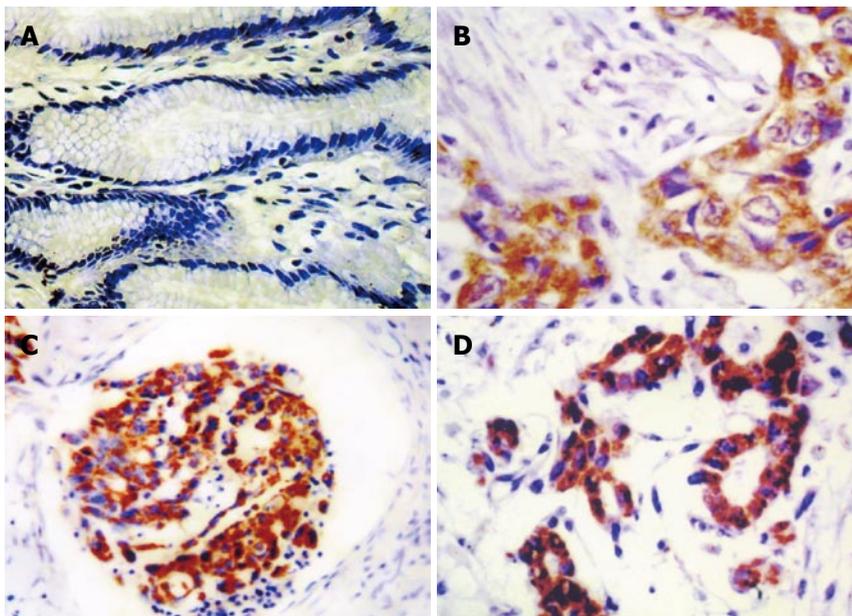


Figure 1 In situ hybridization of uPA mRNA in gastric cancer tissue. **A:** Negative in the plasma of non-tumor gastric mucosa (ISH \times 100); **B:** Positive in the plasma of gastric adenocarcinoma (ISH \times 220); **C:** Positive in plasma of gastric adenocarcinoma with lymphangial cancer embolus (ISH \times 180); **D:** Positive in the plasma of gastric adenocarcinoma with greater peritoneum infiltration (ISH \times 180).

sections were deparaffinized, and then endogenous peroxidase was blocked by incubating the slides with 30 mL/L H_2O_2 for 10 min at 37°C. After being thoroughly washed with distilled water 3 times (2 min each time), the slides were heated in the jar containing antigen retrieval solution (0.01 mol/L citrate buffer, pH 6.0) in an oven at 92-98°C for 15 min for the retrieval of the antigens and cooled to room temperature. After being washed with PBS (0.01 mol/L, pH 7.4) for 5 min, the sections were further blocked by goat serum for 20 min at 37°C to reduce nonspecific antibody binding and then incubated separately with primary antibodies (mouse anti-human VEGF or mouse anti-human CD34) at 4°C overnight. After being washed 3 times (3 min each time) in PBS, the sections were incubated with the biotin-labeled goat anti-mouse IgG at 37°C for 30 min, washed again with PBS, followed by incubation with streptavidin-peroxidase complex for 30 min at 37°C. Staining was visualized with DAB for 10 min at room temperature. Finally, the sections were counterstained by hematoxylin solution. To examine the specificity of immunostaining, PBS was used to replace the primary antibodies as the control.

Results evaluation

The cytoplasm of the uPA and uPAR mRNA appeared as brown in color. Two hundred cells were randomly chosen by microscopy to evaluate the stained cell number against the total cell number in the field. Based on the positive cell number, the criteria were set as follows: negative (-) = less than 10% positive cells or without positive staining; (+) = 11%-50% positive cells; (++) = 51%-75% positive cells; and (+++) = more than 75% positive cells. Based on the VEGF-positive cell number, the criteria were set as follows: negative (-) = no positive staining; (+) = less than 25% positive cells; (++) = 26%-50% positive cells; and (+++) = more than 50% positive cells. The MVD in the carcinoma tissue was calculated as previously described^[8]. Briefly, positive stainings for MVD, in five most highly vascularized areas ('hot spots') in each slide, were counted

in 200 \times fields and MVD was expressed as the average of the microvessel count in these areas. Any endothelial cell or endothelial cluster positive for CD34 (brown yellow staining) was considered to be a single countable microvessel. All 105 cases were divided into high MVD (MVD \geq 54.9/mm²) group and low MVD (MVD < 54.9/mm²) group according to the MVD mean value of 105 cases (54.9/mm²)^[9].

Statistical analysis

Statistical evaluation was performed using χ^2 test or Fisher's exact test to differentiate the rates of different groups, *t*-test was used to analyze quantitative data, and rank sum correlation was analyzed with Spearman's test. The survival rate was estimated by the Kaplan-Meier method and analyzed by log-rank test. $P < 0.05$ was considered statistically significant. SPSS11.0 software for windows was employed to analyze all the data.

RESULTS

uPA mRNA, uPAR mRNA and VEGF protein expression

Twenty cases of non-cancer gastric mucosa had no uPA mRNA and uPAR mRNA expression, while positive staining for uPA and uPAR was observed mainly in cancer cells, but also in number of stromal cells including macrophages and fibroblasts. However, the number of these cells was too small to allow quantitative evaluation of the correlation with clinicopathologic factors. The rates of uPA mRNA and uPAR mRNA positive expression in cancer cells were 58.1% and 66.7%, respectively. The carcinoma cells had brown staining in the cytoplasm and invaded the muscular layer, peritoneum and greater omentum (Figure 1 and Figure 2). Moreover, the front of carcinoma infiltration areas, lymphangial cancer embolus had positive uPA mRNA and/or uPAR mRNA expression (Figure 1 and Figure 2). The positive relationships of each factor with the clinicopathologic parameters are shown in Table 1.

No correlation was found between uPA mRNA and/or

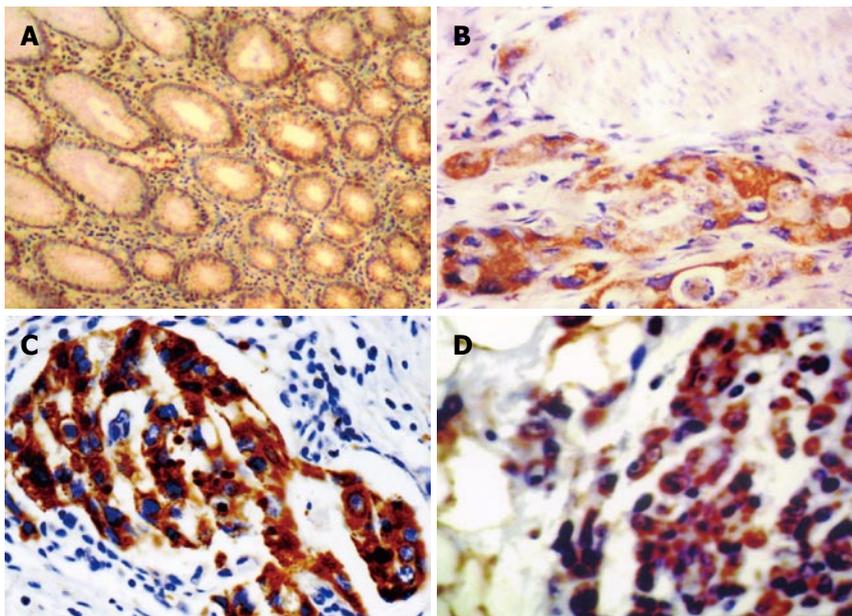


Figure 2 *In situ* hybridization of uPAR mRNA in gastric cancer tissue. **A:** Negative in the plasma of non-tumor gastric mucosa (ISH × 100); **B:** Positive in the plasma of gastric adenocarcinoma infiltrated to muscularis (ISH × 180); **C:** Positive in plasma of gastric adenocarcinoma with lymphangial cancer embolus (ISH × 240); **D:** Positive in the plasma of gastric adenocarcinoma with greater peritoneum infiltration (ISH × 120).

Table 1 Correlation of uPA mRNA, uPAR mRNA and VEGF expression and clinicopathologic parameters in gastric cancer

Clinicopathologic parameters	n	uPA mRNA				uPAR mRNA				VEGF			
		-	+	++	+++	-	+	++	+++	-	+	++	+++
Growth pattern													
Expansive	48	29	8	3	8	21	8	8	11	24	6	8	10
Infiltrative	57	15	8	11	23 ^b	14	7	17	19 ^a	14	5	13	25 ^a
Invasive depth													
T1-T2	44	27	9	2	6	20	11	6	7	31	5	5	3
T3-T4	61	17	7	12	25 ^b	15	4	19	23 ^b	7	6	16	32 ^b
Vessel invasion													
Absent	29	16	8	2	3	14	8	2	5	25	3	1	0
Present	76	28	8	12	28 ^b	21	7	23	25 ^b	13	8	20	35 ^b
Lymph node metastasis													
Absent	35	21	8	3	3	17	8	2	8	27	5	1	2
Present	70	23	8	11	28	18	7	23	22	11	6	20	33 ^b
Distant metastasis													
Absent	63	39	16	1	7	29	14	8	12	36	10	10	7
Peritoneum metastasis	24	2	0	11	11 ^b	2	0	11	11 ^b	0	0	9	15 ^b
Liver metastasis	18	3	0	2	13	4	1	6	7	2	1	2	13

^aP < 0.05, ^bP < 0.01.

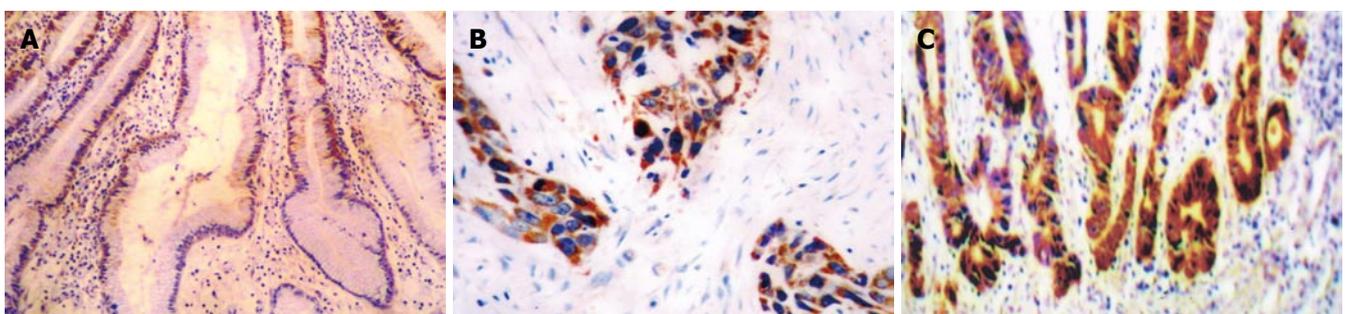


Figure 3 VEGF expression in gastric cancer tissue. **A:** Negative in the plasma of non-tumor gastric mucosa (SP×220); **B:** Positive in the plasma of gastric adenocarcinoma with greater omentum infiltration (SP × 220); **C:** Positive in plasma of gastric adenocarcinoma at the front of the cancer infiltration areas (SP × 180).

uPAR mRNA positive expression and the carcinoma types ($\chi^2 = 1.98, P = 0.78; \chi^2 = 2.82, P = 0.063$), differentiation

($\chi^2 = 2.08, P = 0.083; \chi^2 = 1.08, P = 0.82$), respectively.

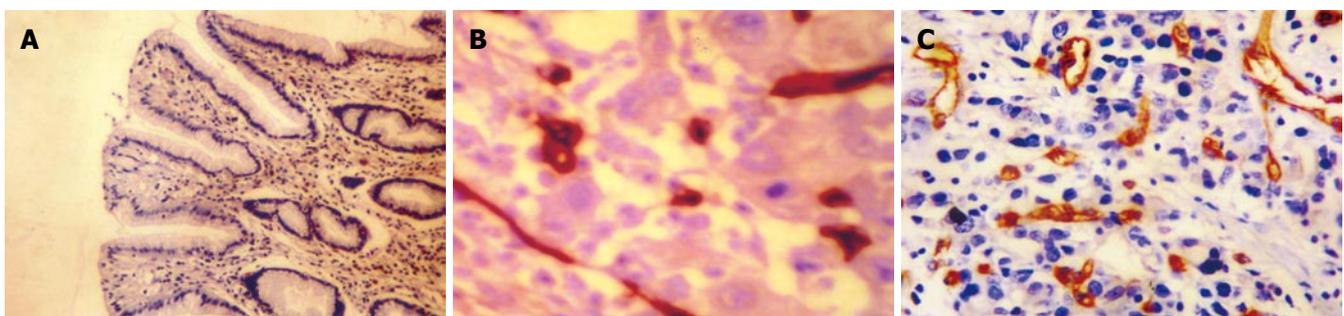
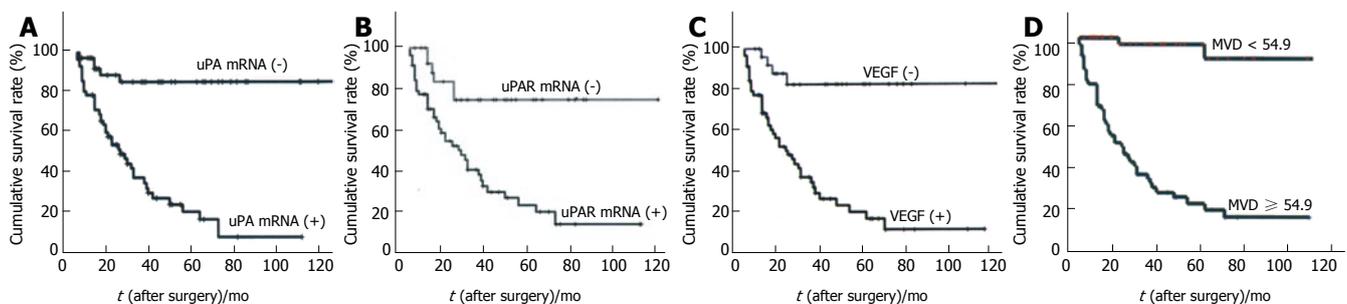
VEGF protein staining was mainly located in the

Table 2 Correlation of MVD with uPA mRNA, uPAR mRNA and VEGF expression in gastric cancer

Expression	<i>n</i>	MVD (<i>n</i> /mm ²)	<i>t</i> value	<i>P</i> value
uPA mRNA			11.25	0.005
+	61	64.89 ± 16.42		
-	44	36.69 ± 14.94		
uPAR mRNA			9.75	0.045
+	70	62.81 ± 19.93		
-	35	39.39 ± 17.29		
VEGF			12.25	0.005
+	67	65.72 ± 14.51		
-	38	35.81 ± 13.36		

Table 3 Correlation of uPA mRNA, uPAR mRNA, VEGF expression and MVD with survival time

Expression	<i>n</i>	Mean survival time (mo)	5-yr survival <i>n</i> (%)	<i>P</i> value
uPA mRNA				
-	44	115.9 ± 7.2	34 (78.9)	0.001
+	61	40.3 ± 6.7	11 (18.2)	
uPAR mRNA				
-	35	107.8 ± 8.8	26 (76.6)	0.002
+	70	49.6 ± 5.7	20 (29.0)	
VEGF protein				
-	38	123.3 ± 9.3	30 (81.0)	0.015
+	67	44.0 ± 7.6	16 (24.1)	
MVD value				
< 54.9	45	85.5 ± 6.8	35 (78.4)	0.006
≥ 54.9	60	38.7 ± 4.8	11 (18.6)	

**Figure 4** Microvessels in gastric cancer. **A:** Negative CD34 in the vascular endothelial cell of non-tumor gastric mucosa (SP × 120); **B:** Positive CD34 in the vascular endothelial cell of gastric adenocarcinoma with MVD < 54.9 (SP × 400); **C:** Positive of CD34 in the vascular endothelial cell of gastric adenocarcinoma with MVD ≥ 54.9 (SP × 120).**Figure 5** Kaplan-Meier survival curves of gastric adenocarcinoma. **A:** With or without uPA mRNA ($P < 0.05$); **B:** With or without uPAR mRNA ($P < 0.05$); **C:** With or without VEGF ($P < 0.05$); **D:** With MVD ≥ 54.9 and MVD < 54.9 ($P < 0.05$).

cytoplasm of tumor cells (Figure 3). About 63.8% gastric carcinoma showed positive expression for VEGF. The positive relationships of the expression of each factor with clinicopathologic parameters are shown in Table 1 (Figure 3). No correlation was found between VEGF protein expression and the carcinoma types ($\chi^2 = 2.21$, $P = 0.078$), differentiation ($\chi^2 = 2.14$, $P = 0.531$). In contrast, twenty cases of non-cancer gastric mucosa had no VEGF protein expression.

Correlation of MVD, uPA mRNA, uPAR mRNA, VEGF expression and survival time

The correlation between MVD, uPA mRNA, uPAR

mRNA and VEGF expression is shown in Table 2. uPA mRNA, uPAR mRNA, VEGF and MVD value within gastric tumor tissue correlated significantly with each other (uPA mRNA and VEGF: $r_s = 0.278$, $P = 0.032$, and MVD: $r_s = 0.199$, $P = 0.042$; uPAR mRNA and VEGF: $r_s = 0.308$, $P = 0.001$, and MVD: $r_s = 0.268$, $P = 0.035$; VEGF and MVD: $r_s = 0.398$, $P = 0.048$; uPA mRNA and uPAR mRNA: $r_s = 0.369$, $P = 0.005$).

The correlation of uPA mRNA, uPAR mRNA expression and MVD value, and survival time were in Table 3 (Figure 4). The survival rate of patients with positive uPA mRNA, uPAR mRNA, VEGF expression and MVD value ≥ 54.9 was significantly lower than that of the patients without these expressions (Figure 5).

DISCUSSION

Degradation of extracellular matrix (ECM) is important in cell migration and tissue remodeling. In cancer tissue, degradation of the ECM is considered to be pro-requisite for cancer invasion and metastasis^[10]. uPA, which belongs to the family of serine proteases, activates plasminogen into plasmin which degrades several components of the ECM, such as fibronectin, laminin and collagen. uPA is released from various cells as an inactive form (pro-uPA). Pro-uPA is converted into an active form after binding to a cell-surface receptor (uPAR). Previous report showed various cell types in which ligand binding to the uPA receptor does induce intracellular phosphorylation on tyrosine^[11], proto-oncogene expression^[12], cell proliferation and cell migration^[13], promoting tumor cell infiltration and metastasis^[14].

In the present study, uPA mRNA and/or uPAR mRNA tended to express more in those patients with serosal invasion, lymph node metastasis, vessel invasion, lymphangial cancer embolus, advanced stage of diseases, and distant metastasis. The results indicated that uPA mRNA and uPAR mRNA was closely related with the clinicopathologic features that reflect the invasion and metastatic potential and prognosis. Up to now, invasion depth, lymph node metastasis or distant metastasis and TNM stage were considered to be the prognostic factors for gastric carcinomas^[15]. uPA mRNA and uPAR mRNA expression statistically related with these factors implied that the invasion and metastasis facilitated by uPA and/or uPAR might indicate prognosis of gastric carcinomas. The results showed that the cancer cells with uPA mRNA and/or uPAR mRNA expression located mainly at invasion front, lymphangial cancer embolus, and involved in gastric wall lymphatic nodule. Our study also revealed that high aggressive cancer with uPA mRNA and/or uPAR mRNA positive expression, which infiltrated muscularis and metastasized to peritoneum, as well as greater omentum, formed cancer glands in the majority. Thus, these results indicated that uPA mRNA and/or uPAR mRNA might be independent biological marker of tumor differentiation. Our data revealed the positive rates of uPA mRNA and/or uPAR mRNA expression in gastric carcinoma with vessel invasion, lymph node metastasis, liver and peritoneum metastasis were significantly higher than that without these clinicopathologic features, and also showed the obvious positive correlation between uPA mRNA and uPAR mRNA, thereby indicating that synergetic high expression of uPA and/or uPAR in tumor cells infiltration area plays a very important role in gastric cancer infiltration and metastasis. Showing that aggressive ability from infiltrating-type growth cancer was stronger than that of expanding-type cancer, our data implied that the more aggressive gastric cancer cells with uPA mRNA and/or uPAR mRNA positive expression degraded ECM strengthenly, thereby facilitating the cancer cells infiltration to the tissue depth, lymphatic and metastatic to the distant organs^[16,17]. So uPA and /or uPAR can be used as a marker of gastric carcinoma infiltration and metastasis.

Both the PA system and VEGF are key factors in

tumor angiogenesis. The PA system degrades the basement membrane and stimulates the migration and progression of endothelial cells in the early phase of angiogenesis^[18]. Beside induction of tumor angiogenesis, VEGF has several additional functions that serve to enhance tumor progression, including enhancing the permeability of tumor vessels^[19] and inhibition of apoptosis of endothelial cells^[20]. An association between the PA system and angiogenesis has been reported. VEGF has been shown to cause up-regulation of uPA and uPAR in endothelial cells. Moreover, a previous study demonstrated that the PA system and VEGF synergistically contributed to liver metastasis of colorectal cancer^[21]. VEGF promotes proliferation of endothelial cells and tube formation after degradation of the ECM by the PA system and/or MMPs^[22,23]. We observed a significant relation of VEGF with both uPA and uPAR in present study. We also observed positive expression of uPA mRNA, uPAR mRNA or VEGF protein in the tumor cells, suggesting that cancer cells with positive expression of uPA, uPAR or VEGF may play an important role in gastric cancer angiogenesis. Interestingly, stepwise analysis demonstrated that uPA mRNA, uPAR mRNA and VEGF protein expression was significantly correlated with MVD. The uPA, uPAR and VEGF produced from gastric cancer cells destroy ECM, which may promote migration of both cancer cells and endothelial cells. On the other hand, cancer cells with high invasive ability may have various malignant potentials, including VEGF production. Since we found a positive correlation between VEGF expression and both uPA mRNA and uPAR mRNA expression, it is possible that the PA system enhances VEGF-induced tumor angiogenesis, which is in agreement with Kaneko *et al.*^[24]. Recently, it has been reported that endostatin inhibits angiogenesis through the down-regulation of the PA system. Thus, the inhibition of uPA and/or uPAR activity may inhibit not only tumor invasion, but also angiogenesis in gastric cancer, and uPA and/or uPAR can be used as a marker of gastric cancer biological behavior.

Previous studies have shown that t-PA and PAI-1 levels are independently associated with survival^[25], and also proved that over-expression of uPA protein is associated with several clinicopathologic features and prognosis^[26,27]. Heiss *et al.*^[28] reported that univariate analysis revealed highly significant inverse correlations between uPA, uPAR and survival time, while multivariate analysis showed PAI-1 was an independent prognostic factor. Lee *et al.*^[29] demonstrated that uPAR level of gastric cancer tissues was correlated with advanced tumor staging and poor survival rates, suggesting that the measurement of uPAR in tumor tissues may be used to predict disease recurrence and the prognosis of gastric cancer. In the present study, survival analysis with the Kaplan-Meier method demonstrated that patients with expression of uPA mRNA and/or uPAR mRNA, VEGF protein had a significantly lower survival rate than those without these. The results suggest that PA system contributes synergistically to tumor invasion, angiogenesis of gastric cancer. uPA mRNA and/or uPA mRNA might be identified as independent prognostic factors.

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Effect of blocking IGF-I receptor on growth of human hepatocellular carcinoma cells

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Abstract

AIM: To study the expression level and localization of insulin-like growth factor -I receptor (IGF-IR) in HepG2 cells and Chang liver cells, and to observe the effect of anti-IGF-IR monoclonal antibody (α IR3) on the growth of HepG2 cells.

METHODS: The expression of IGF-IR in HepG2 cells and Chang liver cells was detected by immunohistochemistry. The influences of α IR3 on proliferation and apoptosis were examined by the 3- (4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay and electron microscopy, respectively. Flow cytometry (FCM) was applied for the analysis of cell cycle and apoptosis was observed under electron microscope.

RESULTS: IGF-IR was located in the membranes of both HepG2 and Chang liver cell lines, and the expression level of IGF-IR was higher in HepG2 cells than in Chang liver cells. Treated with 0.1 μ g/mL α IR3 for 48 h *in vitro*, the cell growth index (GI) of HepG2 cells was significantly higher than that of control (103.41% vs 100%, $P < 0.01$). However, the α IR3 for 24 h at final concentration of 4.0 μ g/mL made the GI of HepG2 cells lower than that of control (93.37% vs 100%, $P < 0.01$). Compared with control, treated with α IR3 for 48 h at final concentrations ranging from 1.0 μ g/mL to 4.0 μ g/mL markedly reduced the GIs of HepG2 cells (97.63%, 97.16%, 95.13%, 92.53% vs 100%, $P < 0.05$ or $P < 0.01$), treated with α IR3 for 72 h at final concentrations ranging from 0.2 μ g/mL to 4.0 μ g/mL decreased the GIs of HepG2 cells obviously (95%, 91.63%, 90.77%, 89.84%, 88.51% vs 100%, $P < 0.01$), and treated with α IR3 for 96 h at final concentrations ranging from 0.5 μ g/mL to 4.0 μ g/mL made GIs of HepG2 cells lower significantly (88.86%, 83.97%, 79.81%, 77.24%, 70.51% vs 100%, $P < 0.05$

or $P < 0.01$). Moreover, treated with α IR3 from 24 h to 96 h at final concentrations ranging from 0.2 μ g/mL to 4.0 μ g/mL reduced the GI of HepG2 cells from 97.63% to 70.51% in a dose- and time-dependent manner. Also, α IR3 treatment for 72 h at final concentration from 0.5 μ g/mL to 2.0 μ g/mL increased the proportion of G₀/G₁ phase cells (61.73%, 67.1%, 83.7%, 76.87% vs 44.47%, $P < 0.01$) and significantly decreased that of S phase cells (28.63%, 25.13%, 15.63%, 23.13% vs 53.17%, $P < 0.01$), in contrast to the proportion of G₂/M phase cells. The apoptotic rates of HepG2 cells were increased more than that of control (7.83%, 16.13%, 21.1%, 37.73% vs 4.13%, $P < 0.01$).

CONCLUSION: The malignant cell phenotype of human hepatocarcinoma cell is related to overexpression of IGF-IR. The blockage of IGF-IR with α IR3 may contribute to the inhibition of proliferation and induction of apoptosis in HepG2 cells.

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Key words: Insulin-like growth factor; Receptor; Monoclonal antibody; Hepatocellular carcinoma cell; Target therapy

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common malignancy in the world and is estimated to cause half a million deaths annually. The incidence of HCC is dramatically increasing in the USA, Europe and Asia, most probably due to the increasing prevalence of chronic hepatitis C, liver cirrhosis and obesity^[1,2]. Unfortunately, the majority of patients suffer from advanced disease at presentation. Curative ablation or resection of HCC, or liver transplantation can be achieved only in a minority of patients. Local tumor destruction, chemoembolization, or systemic chemotherapy are the remaining treatment options in advanced HCC. However, overall survival is poor^[3]. Apart from chemoembolization, which improves survival in well-selected patients with

unresectable HCC, treatment options do not appear to greatly improve overall survival^[4]. Therefore, innovative treatment approaches are urgently needed. Evidence has been accumulated that the insulin-like growth factor-I receptor (IGF-IR) is a promising target for cancer therapy. A great variety of tumors show abnormal, enhanced and/or constitutive expression of IGF-IR. Several reports indicate that IGF-IRs are expressed frequently in HCC^[5], most likely contributing to the aggressive growth characteristics of tumors. Hence, the IGF-IRs are promising targets for innovative treatment strategies in HCC. IGF-IR is a transmembrane heterotetrameric protein, which has two extracellular α -chains and two membrane-spanning β -chains in a disulfide-linked β - α - α - β configuration^[6]. The binding of its ligands (IGF-I, IGF-II) to the extracellular domains of IGF-IR activates its intracellular tyrosine kinase domain resulting in autophosphorylation of the receptor. Activated IGF-IR phosphorylates its substrates and initiates proliferative and antiapoptotic signal transduction pathways that involve phosphatidylinositol-3-kinase and mitogen-activated protein kinase^[7,8]. The high degree of homology to insulin receptor presents a considerable challenge for the development of specific small molecule inhibitors of IGF-IR tyrosine kinase activity. IGFs are known to display mitogenic, transforming, and antiapoptotic properties in various human tumors, including HCC, by stimulating distinct intracellular signaling pathways^[9]. In addition to its role in proliferation of cancer cells, the IGF-IR protects cells from apoptosis caused by growth factor deprivation, anchorage independence, or cytotoxic drug treatment^[10]. Down-regulation of IGF-IR function by antisense and dominant negative techniques reduces the growth and tumorigenicity of several cancer cell lines *in vivo* and *in vitro*, including colon cancer, melanoma, lung carcinoma, ovarian cancer, glioblastoma, neuroblastoma, and rhabdomyosarcoma^[11-17]. IGF-IR is thus an attractive therapeutic target based on the hypothesis that inhibition of IGF-IR function would result in selective apoptosis and growth inhibition of tumor cells^[18]. An effective strategy to inhibit the function of IGF-IR in cancer cells is to use anti-IGF-IR antibodies that bind to the extracellular domains of IGF-IR and inhibit receptor activation. Suppression of the growth of various tumors has been evaluated, but IGF-IR inhibition for the treatment of human HCC remains unexplored. The expression and activity of IGF-IR was shown to be upregulated in HCC cells, which contributes to the process of malignant transformation and growth of liver tumors. Increased expression of IGF may further potentiate the mitogenic effects of IGF in the development of hepatocellular carcinoma^[19]. Hence, in the present study we examined the antineoplastic potency of the IGF-IR inhibitor α IR3 in human HCC cell lines. Our study provides evidence that monoclonal antibody (α IR3) inhibits growth and induces apoptosis and cell cycle arrest in human HCC cells.

MATERIALS AND METHODS

Cells and reagents

HepG2 human hepatocellular carcinoma cells and Chang

liver human normal hepatocytes were from Shanghai Institute of Cell Biology, and grown in RPMI 1640 with 10% heat-inactivated FCS (GIBCO BRL, Carlsbad, California, USA). IGF-IR Ab-1(24-31) was purchased from NeoMarkers, and α IR3 was from Oncogene Science. SABC and DAB kits were purchased from Boster.

Cell culture

HepG2 human hepatocarcinoma cells, normal human hepatocytes were from Shanghai Institute of Cell Biology, Chinese Academy of Sciences. The cells were maintained in RPMI 1640 (Life Technologies Inc.), supplemented with 10% heat-inactivated fetal calf serum (FCS), 0.3% L-glutamine, 1% penicillin/streptomycin solution, which contained 10 000 U/mL penicillin G and 10 mg/mL streptomycin sulfate. Cells were grown as adherent cells in a humidified atmosphere at 37°C in 50 mL/mL CO₂.

Immunohistochemistry

HepG2 and Chang liver cells were passaged onto 22 mm square glass coverslips in a 6-well plate (Nalge Nunc International, Rochester, NY). After 72 h, cells were washed twice with phosphate-buffered saline (PBS) and fixed in methanol (95%) at room temperature for 30 min. After two washes with PBS, cells were incubated in 0.3% H₂O₂ for 30 min to block endogenous peroxidase, then washed three times with PBS. Nonspecific antibody staining was blocked by preincubation with 5% bovine serum albumin (BSA) in PBS for 30 min, then incubated with the primary antibody IGF-IR Ab-1(24-31) at a 1:100 dilution overnight at 4°C. Cells were washed three times for 5 min in PBS. Then they were incubated with the biotinylated goat anti-mouse IgG. After incubation with the secondary antibody, cells were washed three times for 5 min with PBS again and incubated with streptavidin-biotin complex (SABC). After incubation for 30 min at room temperature, cells were then washed four times for 5 min in PBS and detected by using DAB as the substrate. All incubations were carried out in humidified chambers to prevent evaporation. All stainings were compared with negative controls. For each cell line PBS was used in representative negative control instead of primary antibody.

Cell proliferation/Survival assays

The effect of α IR3 treatment on the growth and survival of human cancer cell lines was measured using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay or by cell counting. In brief, when the cultured HepG2 cells reached 70%-80% confluence, the medium was replaced by FCS-free RPMI 1640 and the cells were cultured for 24 h, and then trypsinized with 0.25% trypsin, adjusted to a density of about 1500-3000 cells/well with complete medium. Two hundred microliters of the cells containing the different α IR3 (seven different concentrations ranging from 0.1-4.0 μ g/mL) were plated in 96-well plates and maintained for 24, 48, 72, 96 h separately. At the indicated periods of time, a solution of MTT (20 μ L of a 5 mg/mL solution in PBS) was then added, and the cells were incubated for another 3-4 h. The medium was removed and replaced by 200 μ L of Me₂SO,

and the units of absorption (UA) were measured at 490 nm. Triplicate values were obtained for each well, and each experiment was repeated three times. Growth index (GI, %) = $(UAE/UAc) \times 100\%$ (UAE: average UA value of experimental group; UAc: Average UA value of control group). The proliferation of HepG2 cells was inhibited at $GI < 100\%$; the proliferation of HepG2 cells was stimulated at $GI > 100\%$.

Flow cytometry

After the cultured cells have reached 70%-80% confluency, the medium was replaced by FCS-free RPMI 1640 and the cells were cultured for 24 h, and then trypsinized with 0.25% trypsin. Five $\times 10^4$ cells were plated in 25 mL culture-flasks in 2 mL of RPMI 1640 medium supplemented with 10% FBS, and simultaneously, α IR3 was separately added to cells at final concentrations ranging from 0.5-2.0 μ g/mL. After being treated for 72 h, the five groups of cells were digested and collected by centrifugation. Cells were washed once with 0.01 mol/L PBS (pH 7.2), and fixed in 70% ethanol at 4°C for 18 h. Next, cells were washed once with PBS, digested by Rnase (50 μ g/mL) and stained with propidium iodide (PI, 100 μ g/mL) for 30 min. The cell cycle phases and DNA content were detected and apoptosis was quantified by determining the percentage of PI-stained nuclei in the subdiploid peak in cell samples analyzed by FCM (Coulter, EpicsXL).

Electron microscopy

When the cultured HepG2 cells reached 70%-80% confluence, the medium was replaced by FCS-free RPMI 1640 and the cells were cultured for 24 h, and then trypsinized with 0.25% trypsin, 2.5×10^5 cells were plated in 50 mL culture-flasks in 10 mL of complete medium, and α IR3 was added to cells at final concentration of 1.0 μ g/mL as reported previously^[20]. Being treated for 72 h, the harvested cells were fixed with 2.5% glutaraldehyde in 0.1 mol/L PBS (pH 7.2) for 4 h. After washed in PBS, the cells were treated with 1% osmium tetroxide in 0.1 mol/L PBS overnight at 4°C, washed three times with PBS, dehydrated with a graded series of acetone and embedded in Epon812, which were polymerized at 60°C for 48 h. Ultrathin sections 50-80 nm thick were counterstained with uranylacetate and lead citrate and then observed under transmission electron microscope (EM) (H-600, Japan) at 100 kV.

Statistical analysis

The data were analyzed using one-way analysis of variance followed by least significant difference test (SPSS11.0 statistical software). The results were expressed as the mean \pm standard deviation (SD). A statistically significant difference was considered to be present at $P < 0.05$.

RESULTS

Expression of IGF-IR in HepG2 cells and Chang liver cells

IGF-IR expression was detected in both HepG2 cells and Chang liver cells, and appeared to be localized (Figure 1A and 1B). The positive reaction for IGF-IR was distinctly

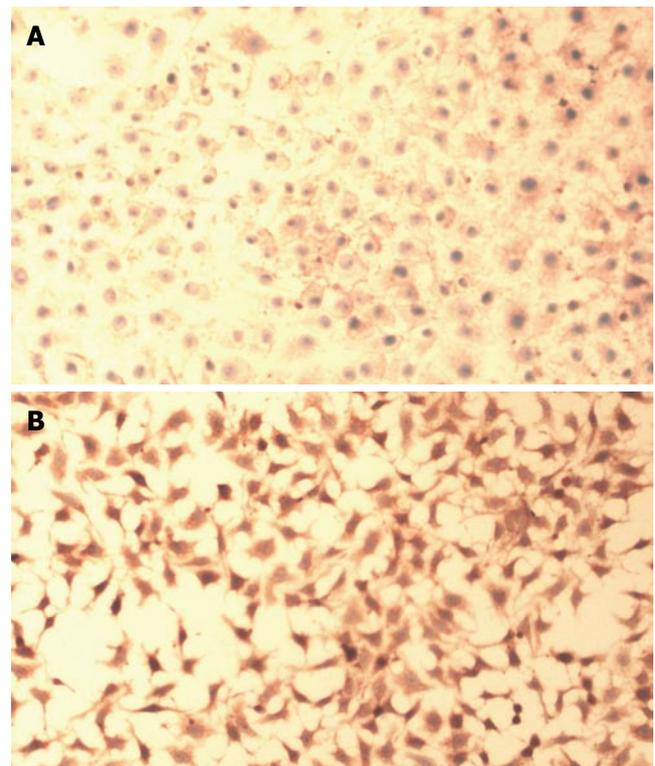


Figure 1 A: Immunohistochemical findings of IGF-IR in Chang liver cells. Chang liver cells showing positive-staining of IGF-IR in the cell membranes of the cells (SABC, original magnification $\times 100$); B: Immunohistochemical findings of IGF-IR in HepG2 cells. HepG2 cells showing stronger positive-staining of IGF-IR in the cell membranes of the cells than that of Chang liver cells (SABC, original magnification $\times 100$).

different between HepG2 cells and Chang liver cells. Compared with Chang liver cell (Figure 1A), a stronger positive reaction for IGF-IR was detected in HepG2 cells (Figure 1B).

Cell growth index (GI) of HepG2 cells

MTT assay was used to investigate the proliferation rates of the cells treated with various concentrations of α IR3 for different periods of time. Unexpectedly, having being treated with α IR3 at final concentration of 0.1 μ g/mL for 48 h, GI of the cells was 104.13%, higher than that of the control group ($P < 0.01$). The result suggests that α IR3 of 0.1 μ g/mL could stimulate HepG2 cells to proliferate. However, after treatment with α IR3 at final concentration of 4.0 μ g/mL for 24 h, GI of the cells was 93.37%, lower than that of the control group ($P < 0.01$). Treated with α IR3 at final concentrations ranging from 1.0-4.0 μ g/mL for 48 h, GI of the different concentration groups was 97.63%, 97.16%, 95.13% and 92.53%, respectively, lower than that of the control group (1.0 μ g/mL group, $P < 0.05$; for the others, $P < 0.01$). After treatment with α IR3 at final concentrations ranging from 0.2-4.0 μ g/mL for 72 h, GI of the different concentrations groups was inhibited by 95%, 91.63%, 90.77%, 89.84%, 88.51% and 86%, respectively, significantly lower than that of the control group ($P < 0.01$). Treated with α IR3 at final concentrations ranging from 0.5-4.0 μ g/mL for 96 h, GI of the different concentration groups decreased by 88.86%, 83.97%, 79.81%, 77.24% and 70.51%, respectively,

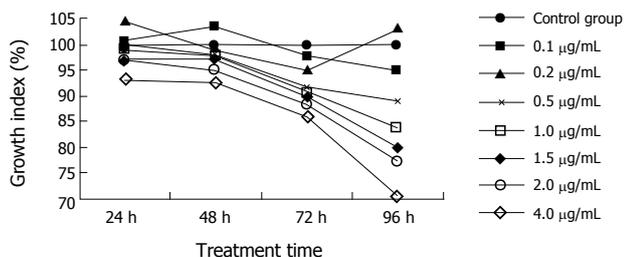


Figure 2 Effects of α IR3 on in vitro growth of HepG2 cells treated with various concentrations of α IR3 for different periods of time. α IR3 of 0.1 μ g/mL could stimulate HepG2 cells to proliferate, while α IR3 inhibited HepG2 cell proliferation in a dose- and time-dependent manner at a concentration ranging from 0.2 μ g/mL to 4.0 μ g/mL.

Table 1 Effect of α IR3 on growth and proliferation of HepG2 cells treated with various concentrations of α IR3 for 24, 48, 72 and 96 h

Group	Cell growth index (GI)			
	24 h	48 h	72 h	96 h
Control	100	100	100	100
0.1 μ g/mL	100.640	103.4133 ^a	97.654	95.032
0.2 μ g/mL	104.716	98.952	94.9961 ^b	103.125
0.5 μ g/mL	99.840	98.175	91.6341 ^b	88.8622 ^a
1.0 μ g/mL	99.041	97.6343 ^a	90.774 ^b	83.9744 ^b
1.5 μ g/mL	97.042	97.1612 ^b	89.8358 ^b	79.8077 ^b
2.0 μ g/mL	96.883	95.1335 ^b	88.5066 ^b	77.2436 ^b
4.0 μ g/mL	93.3653 ^b	92.5313 ^b	86.0047 ^b	70.5128 ^b

^a $P < 0.05$, ^b $P < 0.01$ vs control.

dramatically lower than that of the control group (0.5 μ g/mL group, $P < 0.05$; the others, $P < 0.01$). These results indicate that with the increase of concentration of α IR3 and prolongation of treatment time, the effect of α IR3 inhibiting HepG2 cells proliferation was enhanced also in a dose- and time-dependent manner (Figure 2 and Table 1).

Analysis of cell cycle and apoptosis

To further study the anti-proliferation effect of α IR3 on HepG2 cells, FCM was used. After being treated with α IR3 at final concentration ranging from 0.5-2.0 μ g/mL for 72 h, cell growth was obviously inhibited. The proportion of HepG2 cells in the G₀/G₁ phase increased significantly from 44.47% (of control group) to 83.7%, and in the S phase decreased dramatically from 53.17% (of control group) to 15.63%, but there was no significant change in the proportion of cells in G₂/M phase (Table 1). These results suggest that α IR3 has an anti-proliferation effect on HepG2 cells. To determine whether cell death induced by α IR3 occurred through an apoptotic pathway, genomic DNA fragmentation was assayed as a hallmark of apoptotic cell death. Flow-cytometric analysis was performed to detect the subdiploid (apoptotic) cell population. The apoptotic index (AI) increased in HepG2 cells from 4.13% (of control group) to 37.73%. After treatment with α IR3 at final concentration ranging from 0.5-2.0 μ g/mL for 72 h, the cells exhibited an increase of subdiploid cell population in a dose-dependent manner (Table 2).

Table 2 Effect of α IR3 on cell cycle and apoptosis of HepG2 cells induced at various concentrations

Cell groups	Distribution of cell cycle (%)			Apoptotic index(%)
	G ₀ /G ₁ phase	S phase	G ₂ /M phase	
Control	44.47 \pm 0.4163	53.17 \pm 1.955	2.367 \pm 2.122	4.133 \pm 0.3215
0.5 μ g/mL	61.73 \pm 0.3786 ^b	28.63 \pm 0.5686 ^b	9.633 \pm 0.3512	7.833 \pm 0.4726 ^b
1.0 μ g/mL	67.10 \pm 0.8185 ^b	25.13 \pm 0.7506 ^b	7.800 \pm 0.2000	16.13 \pm 0.3512 ^b
1.5 μ g/mL	83.70 \pm 0.4000 ^b	15.63 \pm 0.3055 ^b	0.6667 \pm 0.5774	21.10 \pm 0.5292 ^b
2.0 μ g/mL	76.87 \pm 1.401 ^b	23.13 \pm 1.401 ^b	0 \pm 0	37.73 \pm 0.1528 ^b

^b $P < 0.01$ vs control group.

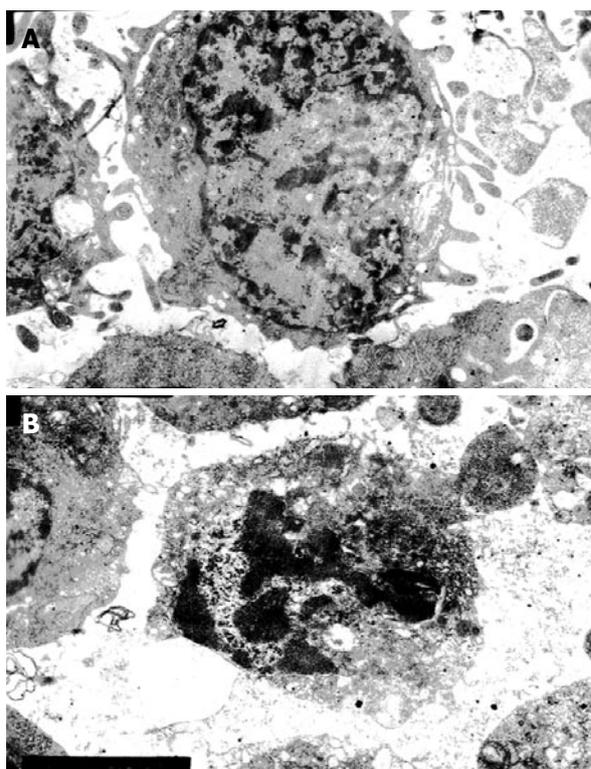


Figure 3 A: Transmission electron micrographs of HepG2 cells from the control group (EM, original magnification \times 6200); B: Transmission electron micrographs of HepG2 cells from the α IR3 group (EM, original magnification \times 6200).

Morphological changes of apoptosis

In HepG2 cells treated with α IR3 at final concentration of 1.0 μ g/mL for 72 h, many characteristic morphological changes of apoptosis were observed under EM. Histological evidence for apoptosis included shrinkage of cells, condensation of cytoplasm, expansion of rough endoplasmic reticulum (RER) and formation of small bubbles, destruction of mitochondria cristae and balloon-shaped mitochondria formation, disappearance or shrinkage of the nuclear membrane, condensation of karyoplasm or chromatin adherence to edge of nuclear membrane with different forms and sizes, formation of apoptotic bodies (Figure 3A and 3B). In the control group, HepG2 cells showed regular nuclei and uniform chromatin distribution within the nuclei, and there were no morphological changes in the mitochondria and other intracellular structures (Figure 3A). These results indicated

that apoptosis could be triggered in HepG2 cells treated with α IR3.

DISCUSSION

IGF-IR is expressed widely in the cell membranes of many cell types, and is essential for normal growth, development, and differentiation and mediates signals for the suppression of apoptosis, enhancement of mitogenesis, and anchorage-independent growth^[21]. Several studies indicate that the number of IGF-IRs on cells is critical in affecting the cell phenotype^[22]; overexpression of IGF-IR may induce transformation from the normal cell phenotype into the malignant cell phenotype. High levels of expression of IGF-IR have been reported in a broad range of human malignancies. Studies of treating IGF-IR as therapeutic target have often been reported. Our immunohistochemical results showed that IGF-IR is expressed in normal human hepatocytes Chang liver cells, suggesting that expression of IGF-IR in the normal tissue and cell plays a pivotal role in cell biological behaviors. Compared with Chang liver cell, the expression level of IGF-IR, which was located in the cell membranes of both cells, was higher in the cell membranes of HepG2 cells, suggesting that overexpression of IGF-IR induces transformation from the normal cell into the malignant cell. In another word, overexpression of IGF-IR is related to the biological behaviors of human malignancies.

Chemotherapy of advanced HCC is still unsatisfactory. Moreover, patients are frequently in poor clinical condition precluding aggressive chemotherapy. Thus, there is a strong need for new, effective and well-tolerated treatment strategies. Some people have focused attention on IGF-IR monoclonal antibody, and the experimental studies of treating some malignancies with the antibody alone or synergistically enhancing the efficacy of cytokines and chemotherapeutic agents have been reported, but its single effect on HCC remains unexplored. In the present study, we investigated the antineoplastic effects of α IR3, a specific IGF-IR inhibitor, on human HCC cells. According to MTT, and FCM assay results, α IR3 inhibited the growth of HCC cells by inducing cell cycle arrest and apoptosis. Surprisingly, α IR3 also stimulated *in vitro* growth of cells in 48 h cultures. This was contrary to our expectation as we undertook these studies to explore the mechanism by which α IR3 inhibits *in vitro* growth of HepG2 cells. To characterize the underlying mechanisms of α IR3's anti-proliferative action on HCC cells, we performed cell cycle analyses. Upon α IR3-treatment the proportion of cells in the G₀/G₁-phase significantly increased, and S-phase significantly decreased in HepG2, suggesting that α IR3 acts at the G₁/S checkpoint. G₁/S cell cycle arrest induced by α IR3 has already been described in hepatocellular carcinoma^[23]. A second mechanism by which α IR3 could inhibit tumor growth is by its down-regulation effect on IGF-IR levels^[24]. α IR3 causes increased endocytosis but may not allow for receptor recycling, causing a net decrease in cell surface receptor levels over time^[24]. Hailey *et al.*^[25] suggest that IGF-IR is also internalized and degraded by a combination of both lysosome-dependent and lysosome-

independent pathways. Induction of apoptosis by α IR3 has been reported previously. In order to confirm this, we used two methods to detect the apoptosis induced by α IR3. Morphological changes of apoptosis in the α IR3 treated cells were seen under an EM, and apoptotic index was measured by FCM, which increased in a dose-dependent manner. Hailey *et al.*^[25] reported that α IR3 inhibited the phosphorylation of AKT, a downstream anti-apoptotic signaling component of the IGF-IR pathway. The activation of both mitochondria-dependent and -independent apoptosis pathways have been reported^[23]. Regulation of cell growth and apoptosis of HCC cells was already shown to be tightly associated with IGF-IR signaling^[26]. It can be speculated that IGF-IR inhibition by α IR3 diminishes mitogenic inputs of the IGF receptor system in HCC cells. Sachdev *et al.*^[24] reported that 250 nmol/L chimeric humanized single-chain antibody (scFv-Fc) activated IGF-IR and downstream signaling pathways, and stimulated the *in vitro* monolayer and anchorage-independent growth of MCF-7 cells, but when treated with 500 nmol/L scFv-Fc, the activity of IGF-IR and *in vitro* proliferation of MCF-7 cells were inhibited. It has been reported that the inhibitory or stimulatory behavior of some antibodies may be dependent on cell surface receptor number^[27]. Surprisingly, in our experiments, lower dose of α IR3 stimulated *in vitro* proliferation of HepG2 cells in 48 h cultures instead of inhibiting HepG2 cells. The mechanism by which lower dose of α IR3 stimulated *in vitro* proliferation of cells needs to be explored further. The mechanisms by which α IR3 inhibited *in vitro* growth of HepG2 cells were through inhibition of the IGF-IR and downstream signaling pathways, inducing cell cycle arrest and apoptosis, IGF-IR level down-regulation, and IGF-IRs degradation; but how the lower dose of α IR3 stimulated *in vitro* proliferation of HepG2 cells is unclear.

Although efficacious in our *in vitro* models of HCC, α IR3 as monotherapy may not be effective in cancers displaying mitogen dependent proliferation. Nevertheless, α IR3 has been shown to synergistically enhance the efficacy of chemotherapeutic agents^[20]. The underlying mechanisms include interference with damage repair mechanisms, maintenance of chemotherapy-induced apoptosis^[28], and the overcoming of growth factor-mediated resistance to chemotherapy^[29]. Thus, α IR3 may well be of clinical benefit for HCC patients, either as single agent or when added to conventional chemotherapy. To conclude, our study provides evidence that the IGF-IR inhibitor α IR3 inhibits the growth of human hepatocellular cancer cells by inducing cell cycle arrest and apoptosis, or stimulates proliferation of the cells. Monoclonal antibody may qualify for the development of targeted therapies for HCC. Further studies are needed to investigate the antitumor effects of monoclonal antibody combined with conventional chemotherapy on HepG2 cells.

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Effects of adenoviral-mediated gene transduction of NK4 on proliferation, movement, and invasion of human colonic LS174T cancer cells *in vitro*

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Abstract

AIM: To investigate the inhibitory effects of a recombinant adenovirus vector that expresses NK4, a truncated form of human hepatocyte growth factor (HGF), on human colonic adenocarcinoma cells *in vitro* to establish a basis for future NK4 gene cancer therapy.

METHODS: Cells from the LS174T human colonic adenocarcinoma cell line were infected with recombinant adenovirus rvAdCMV/NK4 and the effects of the manipulation on tumor cell proliferation, scatter, migration, and basement membrane invasion were assessed. Cells infected with a recombinant adenovirus vector (Ad-LacZ) expressing β -galactosidase served as the controls.

RESULTS: We found that rvAdCMV/NK4 expression attenuated HGF-induced tumor cell scatter, migration, and basement membrane invasion ($P < 0.05$), but did not inhibit tumor cell proliferation.

CONCLUSION: HGF-induced LS174T tumor cell scatter, migration, and invasion can be antagonized by the recombinant NK4-expressing adenovirus.

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Key words: Human colonic adenocarcinoma; NK4; Hepatocyte growth factor; Adenoviral vector

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INTRODUCTION

Hepatocyte growth factor (HGF), a glycoprotein consisting of 728 amino acids with a 69 kD α -chain and a 34 kD β -chain, affects tumor metastasis^[1,2]. HGF binds to c-Met, the only HGF receptor, and causes a cascade of enzyme catalyzed reactions, triggers signal transduction and the corresponding biological effects^[3]. In the normal condition, the paracrine influence of HGF between the epithelial and interstitial cells is strictly controlled. However, when abnormal HGF/c-Met signaling occurs, such as there is a mutation of the c-Met gene, over-expression of HGF and/or c-Met, or there is co-expression of HGF and c-Met within the same cells, c-Met may be persistently over-expressed and thereby exhibit a high-level of self-phosphorylation. All these biological activities, in turn, facilitate the occurrence, growth, infiltration, metastasis, and angiogenesis of many kinds of tumors^[4,5]. The HGF/c-Met signaling pathway has been thought to play an important role in the production of many kinds of human tumors^[6,7]. Studies discovering the ways to block this pathway will provide new targets for the anti-metastasis tumor treatment.

NK4, a specific antagonist of HGF discovered by Date *et al*^[8] in 1997, is a fragment from the HGF molecule that has been cut by elastase between the 478th and the 479th amino acid. NK4 derives its name from its structure: It is composed of the NH₂-terminal hairpin domain and four subsequent kringle domains of the α -chain of HGF. NK4 contains 447 amino acids, and the molecular weight is about 50 kD. Although NK4 competes with HGF for c-Met receptor binding, it cannot activate the c-Met receptor and induce its phosphorylation. Consequently, NK4 suppresses the interaction between HGF and c-Met, interrupts the HGF/c-Met signaling pathway, and thereby inhibits HGF-induced invasion and metastasis of tumor cells^[9,10].

In this study we used the replication-defective recombinant adenovirus rvAdCMV/NK4 expressing NK4 gene^[11] to investigate the anti-tumor effects of NK4 in LS174T human colonic adenocarcinoma cells. The objective of this study was to explore whether NK4 may be used as a human colonic tumor treatment- a possibility that would require an experimental basis for the clinical application of manipulations of the NK4 gene product.

MATERIALS AND METHODS

Virus and cell culture

The construction and identification of the recombinant adenovirus rvAdCMV/NK4 with E1-E3 deletions have been described previously^[11]. The recombinant adenovirus expressing β -galactosidase (Ad-LacZ) was generated in our laboratory using the same vector (data not published). These two viruses were replicated in a HEK293 human embryonic kidney cell line (American Type Culture Collection, Manassas, VA), which was maintained in DMEM medium (Invitrogen, Carlsbad, CA) supplemented with 100 mL/L fetal bovine serum (FBS) (Invitrogen). Culture titers were 8.9×10^{12} infection focus unite (ifu)/L and 6.4×10^{12} ifu/L, respectively. LS174T human colonic adenocarcinoma and MRC-5 human fetal lung fibroblast cell lines were purchased from the Shanghai Institute of Cell Biology at the Chinese Academy of Sciences and cultured in RPMI 1640 medium (Invitrogen) supplemented with 100 mL/L FBS, 100 U/mL penicillin, and 100 mg/L streptomycin (Invitrogen).

Western blots

Total cellular proteins were extracted and aliquots were separated by 12% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The blots were incubated with rabbit anti-human c-Met polyclonal antibody [1:250] (Zymed Laboratories, San Francisco, CA) or goat anti-human HGF- α polyclonal antibody [1:500] (Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h at room temperature, followed by the addition of alkaline phosphatase (AP) conjugated secondary antibodies [1:5000] (Pierce, USA). AP was reacted with BCIP/NBT to visualize the bands.

Adenovirus transduction efficiency analysis

LS174T cells were seeded in 24-well plates until they reached 80% confluency, at which time they were infected with 1, 10, 25, 50 or 100 multiplicity of infection (MOI) with Ad-LacZ for 48 h. The cells were washed with phosphate buffered saline (PBS) and fixed in 100 mL/L formaldehyde for 10 min. Fixed cells were then stained with X-gal overnight at 37°C. The number of positive cells in each well was counted by viewing under a light microscope. Transduction = (the number of positive cells/number of total cells) \times 100%.

Cell proliferation assay

Two thousand five hundred LS174T cells/well were seeded in 96-well plates in triplicate and divided into a control group, an Ad-LacZ group, and a rvAdCMV/NK4 group.

The cells were cultured for 24 h (d 0), then mock infected or infected with viruses at 50 MOI for 1 h. The medium was then replaced with RPMI 1640 containing 100 mL/L FBS, and the cells were further cultured and harvested on d 0, 1, 3, and 5. Cell proliferation was analyzed using the CellTiter 96 Aqueous One Solution Regent (Promega, Madison, WI) according to the manufacturer's protocol.

Cell scatter assay

LS174T (2.5×10^3 cells/well) was seeded in 12-well plates. After culturing for 4 to 7 d at 37°C, the medium was replaced with 20 mL/L FBS RPMI 1640 medium. The cell cultures were divided into a control group, an Ad-LacZ group (50 MOI), and a rvAdCMV/NK4 group (50 MOI) and treated with recombinant human HGF (PeproTech EC, Rocky Hill, NJ) at a final concentration of 10 μ g/L for 48 h. The cell colony scattering was observed and photographed.

In vitro scratch wound healing assay

Cell migration was measured by an *in vitro* scratch wound healing assay as described elsewhere^[12]. Briefly, after LS174T cells were infected by 50 MOI of rvAdCMV/NK4 or Ad-LacZ for 48 h, the cells were trypsinized and adjusted to a density of 2×10^9 cells/L, then seeded in 12-well plates. Monolayer cells were scratched with a sterile pipette tip, and the cell migration was evaluated by counting the number of cells that had migrated from the wound edge after culturing for 24 h in 20 mL/L FBS-supplemented RPMI 1640 medium.

Invasion Assay

Invasion assay was carried out as previously described^[13]. Matrigel, an artificial matrix gel extracted from mice EHS sarcoma (purchased from the Department of Cellular Biology, Center for Health Sciences, Peking University, Beijing, China), was diluted by DMEM medium to 1 g/L. Polycarbonate membranes of the upper wells in a Transwell chamber (Corning Costar No. 3422) were loaded with 50 mL of Matrigel gel and exposed to UV for 30 min. Serum free medium was added to the chamber for polymerizing at 37°C for 30 min before using. LS174T cells that had been transduced with rvAdCMV/NK4 or Ad-LacZ at an MOI of 50 for 72 h were adjusted to a density of 1.5×10^9 cells/L. Cell suspension (100 μ L) was seeded on the upper wells and 600 μ L of serum-free RPMI 1640 media, with or without 10 μ g/L HGF, was added to the lower wells of the Transwell chamber. After cultivating for 72 h, the cells that degraded from the Matrigel and migrated through the 8 μ m pores of the membrane at the bottom of the upper wells to the opposite side of the membrane were stained with Hematoxylin and Eosin and counted. Five microscopic fields (\times 200) were randomly selected for cell counting. The inhibitory rate = [(the number of invading cells in control group-the number in treated group)/the number of invading cells in control group] \times 100%.

An inhibitory rate of more than 30%, $P < 0.05$, when compared with the group in which tumor cells in the upper wells and lower wells contained HGF, was considered to be evidence of an anti-invasion effect.

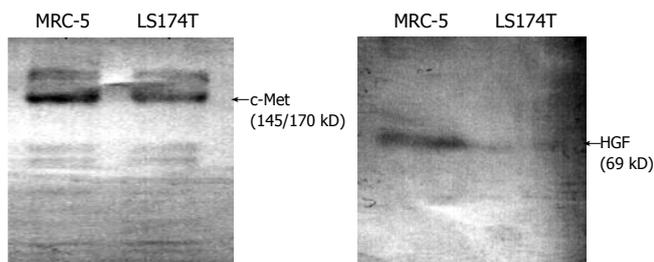


Figure 1 c-Met and HGF expression by Western blot.

Statistical analysis

All data are expressed as means \pm SD. Statistical comparisons were made using the Student's *t*-test. $P < 0.05$ was considered significant.

RESULTS

Expression of c-Met in the LS174T cell line

In this study we first examined the expression of c-Met, in order to test the reliability of using the colon cancer cell line LS174T as a study model. Western blot results showed that two specific bands were detectable in both LS174T and MRC-5 (Control): One was the 145 kD β -subunit of c-Met protein and the other was the 170 kD c-Met protein existing in the heterogeneous dimer form. This finding suggests that LS174T cells could be used as target cells of HGF and thus employed in further experimental studies. Expression of HGF was detected in MRC-5 cells, but was not detected in LS174T cells (Figure 1).

Adenovirus transduction optimization and NK4 gene expression

In order to optimize the gene transduction mediated by the adenovirus, we used the recombinant adenovirus Ad-LacZ, containing the report gene β -galactosidase, to infect LS174T cells at different MOI. We found that the infection rate increased with increasing MOI in a dose-dependent manner (data not shown). When the infection intensity reached 50 MOI, Ad-LacZ achieved a LS174T cell infection rate of more than 90% without pathological changes. Since overdose recombinant adenovirus infection will hurt cells and disturb cell growth, the optimal infection dose in all of our experiments was set as 50 MOI. rvAdCMV/NK4 infection of LS174T cells was confirmed by Western blot analysis. A specific NK4 protein band at 50 kD was detected, demonstrating that efficient expression of the NK4 gene mediated by the adenovirus was achieved in the LS174T cells (Figure 2). Thus, rvAdCMV/NK4-infected LS174T cells were found to be suitable for our experiments.

rvAdCMV/NK4 suppression of LS174T cell proliferation

We measured the effect of rvAdCMV/NK4 transduction on proliferation of LS174T cells. The proliferation rate of the tumor cells infected by rvAdCMV/NK4 was similar to that of the control group and the Ad-LacZ group ($P > 0.05$). As summarized in Figure 3, these data suggest that expression of the NK4 gene mediated by the adenovirus

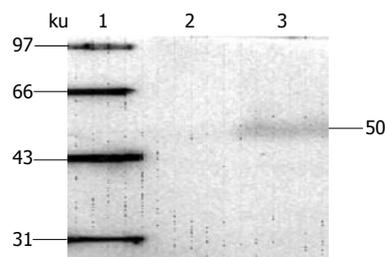


Figure 2 Recombinant adenovirus rvAdCMV/NK4 mediated NK4 expression in LS174T cells. Notes: 1: Protein molecular weight markers; 2: Mock; 3: rvAdCMV/NK4.

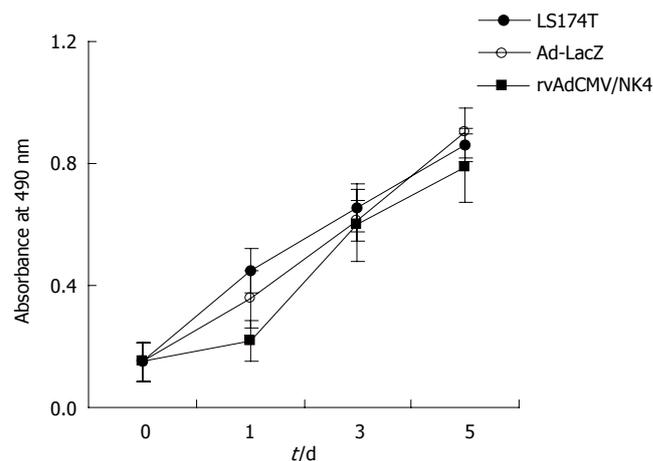


Figure 3 Effects of NK4 expression on LS174T cell proliferation *in vitro*.

had no suppressive effect on LS174T cell proliferation.

Inhibition of LS174T cell scattering by rvAdCMV/NK4

Since HGF facilitates cell colony scatter, we designed this experiment to determine whether NK4 has an inhibitory effect on cell scattering. The results showed that intercellular adhesion among untreated LS174T cell colonies grown for 4 to 7 d was strong and that cells were not prone to scatter. But after HGF treatment, adhesion was relatively weak and the cells scattered in a spider-like pattern. In contrast, this HGF-effect was inhibited in rvAdCMV/NK4-infected cells. However, sole Ad-LacZ or rvAdCMV/NK4 infection did not influence intercellular adhesion, indicating that NK4 could specifically antagonize HGF-induced cell scattering (Figure 4).

Inhibition of LS174T cell movement by rvAdCMV/NK4

We performed a scratch wound healing assay to determine whether NK4 expressing recombinant adenovirus could attenuate HGF-induced cell motility. Without HGF treatment, the number of Ad-LacZ or rvAdCMV/NK4 infected LS174T cells that migrated into the scratch wound area were similar with that of uninfected LS174T cells. However with HGF treatment, we observed that less NK4-expressing cells migrated to the scratch wound area than control cells did, indicating that HGF activity of promoting tumor cell movement was antagonized by NK4. The number of cells migrating into the scratch wound areas of each group after HGF treatment was: $28.8 \pm 7.1/\text{mm}^2$

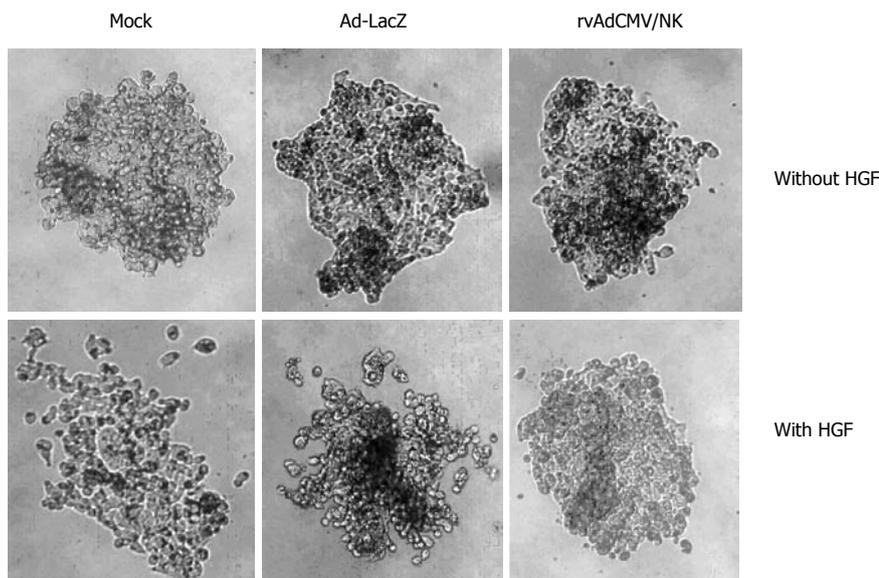


Figure 4 Scattering assay for LS174T cells after treatment with rvAdCMV/NK4 or Ad-LacZ.

in the control group; $29.3 \pm 9.4/\text{mm}^2$ in the Ad-LacZ group ($P > 0.05$ vs control group); and $10.2 \pm 3.6/\text{mm}^2$ in the rvAdCMV/NK4 group ($P < 0.05$ vs control group, Figure 5). These results suggest that the recombinant NK4 expressing adenovirus selectively antagonized HGF-induced cell movement.

Inhibition of LS174T cell Invasion by rvAdCMV/NK4

Tumor cell invasion of the basement membrane is a key process during metastasis. Matrigel, which is a kind of artificial matrix gel extracted from mouse EHS sarcoma, forms a membrane structure similar to the natural basement membrane structure in serum-free medium. The malignant invasion capacity of cells is revealed by their ability to penetrate into the filter membrane under the influence of an inductive chemo-attractant. The influence of specific factors on tumor cell invasive capacity can be observed *in vitro* by counting the number of cells that penetrate the filter membrane. The ability of untreated LS174T cells to degrade and penetrate the basement membrane was poor, but was improved when HGF was present in the lower chamber. The number of Ad-LacZ or rvAdCMV/NK4 infected LS174T cells that penetrated through the membrane without HGF in the lower chamber was similar to that of non-infected LS174T cells ($P > 0.05$). However, the invasive capacity of LS174T cells decreased significantly when HGF was present in the lower chamber (inhibitory rate 71.6%) after infection with rvAdCMV/NK4; meanwhile the inhibitory rate was only 7.3% in the Ad-LacZ treated group. This difference ($P < 0.05$) suggests that NK4 can specifically inhibit HGF-induced LS174T cell invasion (Figure 6).

DISCUSSION

Despite considerable advances in anesthesiology and surgical techniques as well as the development of new radiotherapy and chemotherapy strategies, the survival rate associated with colonic cancer has not been greatly improved. Tumor metastasis, including local metastasis to

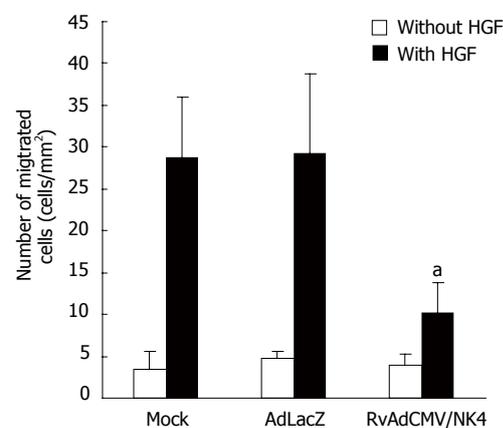


Figure 5 Scratch wounding healing assay for LS174T cells treated with rvAdCMV/NK4 (mean \pm SD). ^a $P < 0.05$ vs Ad-LacZ or Mock (with HGF).

peripheral viscera and distant metastasis, is thought to be an important factor in determining survivability. It is reported that 15% to 35% of patients suffering from primary colonic cancer had liver metastasis when their primary tumors were diagnosed. Even among those patients who accept radical treatment for colonic cancer, 25% will have liver metastasis^[14]. Accordingly, the prognosis of patients suffering from colonic cancer is primarily associated with the occurrence of metastasis, especially to the liver. Thus inhibition of metastasis will markedly improve the prognosis of colon cancer patients.

Changes in oncogenes and anti-oncogenes within cells contribute to tumorigenesis, but tumor metastasis behavior is primarily regulated by extracellular activated growth factors. Stroma cells can regulate tumor cell motility by releasing various cytokines, and consequently may initiate tumor metastasis^[15-17]. HGF is one such important factor, released primarily by stroma fibroblasts, and initially identified and cloned as a potential liver cell mitogen^[18,19]. HGF has also been found to facilitate cell movement and extracellular matrix breakthrough, resulting in the scatter of many kinds of tumor cells^[4,20-22] and stimulating tumor cell prolifera-

tion and stroma cell angiogenesis^[23]. All of the effects of HGF are realized through its binding with the c-Met receptor, and c-Met receptors are over-expressed in many kinds of tumor cells^[24-26]. In this study we used the recombinant NK4 expressing adenovirus to infect the LS174T human colonic cancer cell line. Our results showed that NK4 could efficiently antagonize the effects of HGF, that is, it could inhibit the migration, scatter, and invasion of tumor cells. This suggests that NK4 is capable of blocking the HGF/c-Met signaling pathway and thereby may play an important role in antagonizing the invasion and metastasis of colonic cancer.

Highly malignant LS174T cells were harvested from poorly differentiated epithelial adenocarcinoma tissue. Western blot analysis indicated that c-Met protein was expressed in the LS174T cells. These findings demonstrate the feasibility of using recombinant adenovirus expressing NK4 to block the HGF/c-Met signaling pathway as a clinical intervention for colonic cancer. We did not detect HGF expression in the LS174T cells; however, the c-Met receptor was triggered clearly after HGF treatment, producing enhanced scatter, motility and invasion. These findings indicate that the HGF treatment was sufficient for this study. In *in vitro* cell proliferation experiments, rvAdCMV/NK4 had no effect on tumor cell proliferation. This is consistent with the studies of Kubota *et al.*^[27] and Saimura *et al.*^[28] indicating that the virus itself does not influence scatter and invasion.

A close relationship between the ability of tumor cells to move and their invasion capacity has been demonstrated^[29]. Thus suppression of tumor cell movement would be expected to inhibit invasion and metastasis^[30]. Initially, tumor cell movement appears as a scattering of the cells away from one another. We used a scatter assay and a scratch wound healing assay to reveal the inhibitory effects of NK4 on HGF. HGF reduced the intercellular homogeneous adhesion of LS174T cells; such as LS174T cells scattered and migrated. These effects were inhibited by rvAdCMV/NK4; however rvAdCMV/NK4 alone (without the presence of HGF) did not have any biological effects on LS174T cells, indicating that the effects of NK4 were specific for the HGF signal. The findings of Parr *et al.*^[31] was shown that the growth factor TGF- β was able to facilitate tumor cell scattering in the presence of the NK4 protein and provide further evidence for the conclusion that NK4 specifically suppresses the influence of HGF.

Once tumor cells fall off the original tumors they invade the extracellular matrix of the host, and then penetrate the lymphatic system or the blood vessels, resulting in distant metastasis. The Matrigel material is similar to the extracellular matrix and therefore can be used to simulate tumor cell penetration into the cellular matrix around blood vessels. LS174T cells were able to degrade the Matrigel and migrate across the 8 μ m membrane pores under the inductive influence of the chemo-attractant HGF. rvAdCMV/NK4 weakened the HGF-induced cell capacity for penetrating the basement membrane, but rvAdCMV/NK4 alone did not produce this effect. Dormancy therapy aimed at preventing angiogenesis has become a hot topic of discussion regarding with anti-tumor treatment^[32]. We did not investigate the antagonizing effect of NK4 on

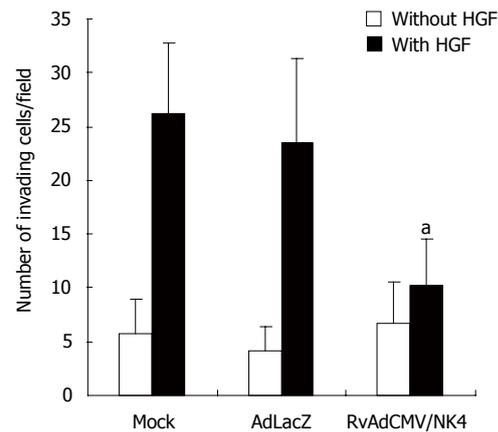


Figure 6 Invasion assay for LS174T cells treated with rvAdCMV/NK4 (means \pm SD). ^a $P < 0.05$ vs Ad-LacZ (with HGF).

HGF-induced angiogenesis, which plays an important role in tumorigenesis and distant metastasis. However, recent studies have shown that the anti-angiogenesis effects produced by NK4 appear to be the result of not only a blockade of HGF-mediated effects, but also a blockade of effects mediated by vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF)^[33,34]. Thus NK4 may be considered as a multi-potential anti-tumor therapeutic protein. Because NK4 has the potential to impact many aspects of tumorigenesis, it will be great applicable in the future clinical treatment of tumors.

In conclusion, this study demonstrated that adenoviral-mediated NK4 inhibits the invasion and metastasis of LS174T colonic cancer cells by blocking the HGF/c-Met signaling pathway *in vitro*. These findings establish an experimental basis for further studies *in vivo*.

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Characterization of *flgK* gene and FlgK protein required for *H pylori* Colonization—from cloning to clinical relevance

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CONCLUSION: FlgK encoded by *flgK* is important for flagella formation and *H pylori* motility. Deficiency in FlgK or an enhanced serological response to r-FlgK can interfere with *H pylori* colonization. FlgK of *H pylori* could be a novel target for vaccination.

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Key words: *H pylori*; Colonization; Isogenic mutant; BALB/c mice; Flagella; Vaccine.

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Abstract

AIM: To characterize the role of *flgK* and its protein product in *H pylori* colonization.

METHODS: The PCR cloning method identified the *flgK* gene. An isogenic *flgK* mutant was constructed by gene replacement and confirmed by Southern blot analysis and PCR analysis. The recombinant FlgK protein (r-FlgK) was purified. Electron microscopy (EM) was applied to demonstrate the flagella of *H pylori*. An in vitro motility test was assessed in semisolid medium. The densities of *H pylori* colonization with either the wild-type strain or its *flgK* mutant were compared among BALB/c mice with or without pre-immunization with r-FlgK. The serological responses to r-FlgK were analyzed for 70 clinical patients with different densities of *H pylori* colonization.

RESULTS: From a duodenal ulcer strain, the *flgK* gene was cloned and it contained 1821 bp, with a 95.7% identity to the published sequences. No flagella were observed under EM for the mutant strain, which had a loss of motility. *H pylori* density was lower in the BALB/c mice inoculated by the mutant or with pre-immunization with r-FlgK compared to unimmunized mice or mice inoculated by the wild-type strain ($P < 0.05$). In the *H pylori*-infected patients, the serological responses to r-FlgK were uniformly low in titer.

INTRODUCTION

H pylori is now well established as a causative agent and predisposing factor for peptic ulcers and even gastric malignancy^[1-5]. However, the actual mechanism by which gastroduodenal diseases develop in response to *H pylori* infection remains unknown. The putative pathogenic factors of *H pylori* are categorized as colonization, persistence, and disease inducing factors^[4]. Colonization in the host is a prerequisite of bacterial infection and subsequent pathogenesis. The putative pathogenic factor of *H pylori* for colonization could be the possible target for preventive or even therapeutic strategy, such as vaccination.

Besides urease production and adhesin (to contact with the gastric epithelium), motility enhanced by unipolar flagella is essentially required for *H pylori* colonization^[5-7]. The motility of *H pylori* is provided by two to six polar, sheathed flagella, the filaments of which consist of two flagellin types, FlaA and FlaB^[5,6]. The *in vitro* experiments with *flaA* and *flaB* isogenic strains disclosed that both genes are required for full motility for colonization^[7]. Moreover, the establishment of persistent *H pylori* infection in a mouse model requires full motility and the presence of both flagellins^[8]. Another *H pylori* *fliD* gene, encoding a 76 kDa HAP-2 homologue, was also disclosed to be required for the assembly of flagellar filaments and motility^[9]. Infection of mice with a *fliD* mutant of *H pylori* SS1, a mouse-adapted strain, demonstrated that the FliD protein is necessary for colonization^[9]. Therefore, flagellar genes and their associated proteins should be potential tar-

gets for therapy and vaccine development against *H pylori* infection.

However, there are several genes related to the flagella in *H pylori*. Besides *flaA*, *flaB*, *flaE*, and *fljD*, the other flagellar structural proteins, such as *flgK*, are not well understood. This study thus describes the molecular cloning and characterization of the *H pylori flgK*. Applying the isogenic mutant of *flgK* and the recombinant FlgK protein of *H pylori*, this study has introduced both the *in vitro* and *in vivo* assay to define the role of *flgK* and its associated FlgK protein for *H pylori* colonization. Furthermore, this study has elucidated the clinical relevance of the anti-FlgK serological response to the colonization density of *H pylori* in humans.

MATERIALS AND METHODS

Bacterial strains and plasmids

The *H pylori* isolate (hp250) from a Taiwanese patient with duodenal ulcer was selected for the cloning of the *flgK* gene. The *Escherichia coli* strains used were DH5 α and JM107. The *E. coli* BL21 strain was used to express the FlgK protein. Plasmid pZero-2 (Invitrogen, Carlsbad, CA) was used to clone the *flgK* gene. Plasmid pET30b (Novagen, Madison, WI) was used to express the FlgK protein. The growth and culture conditions of *H pylori* were described previously^[10].

Construction of *flgK*-mutant

Part of the *flgK* gene was amplified by PCR using *H pylori* hp250 DNA as a template with the first primer (5' CGGGATCCCGTCGCCACATCAAAATTTCCC 3') in the *flgK* gene and the second primer (5' GCTC-TAGAGCTTCACTCAACACTTCTTA CACC 3') designed to be complementary to *flgK*. PCR was performed in a DNA thermal cycler (Perkin-Elmer Corporation, Norwalk, Conn.) that had been programmed for 30 cycles of 1 min at 94°C, 1 min at 60°C, and 2 min at 72°C. Following amplification, the 1.4 kb PCR product was digested with *Bam*HI and *Xba*I (New England Biolab, Beverly, MA) and ligated to pZero-2. The 0.8 kb chloramphenicol resistance cassette (*cat*) was inserted into an *Eco*RI site to construct plasmid pMW336. Analyzing digestion patterns obtained with appropriate endonucleases and DNA sequencing confirmed the construction.

Transformation

E coli and *H pylori* was transformed based on the methods applied before^[11,12], and the selection for chloramphenicol resistance was done with 5 μ g/mL.

FlgK expression and purification

The *flaK* gene was amplified by PCR using the sense primer 5'GGGGATCCAATGGGCGGGATCTTATC3' and the antisense primer 5'CGCTCGAGTTATTGTT TAATCCCCAA3'. The PCR product was purified and cloned into the pT7 blue T vector (Novagen) and then sub-cloned to the pET30b vector digested with *Bam*HI and *Xba*I. The recombinant plasmid was transformed into the *E. coli* BL21 strain. Cells were grown at 37°C for

2-3 h in LB medium to an A_{600} up to 0.4-0.6. Isopropyl- β -D-thio-galactopyranoside (1 mmol/L) was added to the culture, and it was further incubated at 30°C for 4 h to induce protein production. Cells were harvested by centrifugation and lysed by a French press. The supernatants were collected and went through the Ni²⁺-chelate chromatography (Amersham Biosciences, Piscataway, NJ). r-FlgK was eluted using a linear gradient of 60 mM-1 M imidazole (Merck, Rahway, NJ) with a flow rate of 60 mL/h, and 1-ml fractions were collected. SDS-PAGE was performed to analyze the proteins in the soluble fractions. The desired proteins contained in the gel slices were identified by amino acid sequencing (Applied biosystems 477A autosequencer) after the proteins had been eluted. The final proteins were stored at -70°C.

Preparation of anti-r-FlgK antibody

Rabbits were injected intramuscularly with 1 mg of r-FlgK mixed with complete Freund's adjuvant; four subsequent immunizations with 500 μ g of r-FlgK mixed with incomplete Freund's adjuvant were given at 2-wk intervals. The serum anti-r-FlgK titers were determined by enzyme-linked assay seven days after the final boost.

ELISA for mice and human serum

Wells of microtiter plates (Dynatech) were coated with r-FlgK and blocked with bovine serum albumin. Serially diluted patient's serum was then added to the wells and incubated at room temperature for 2 h. Goat-anti-rabbit-IgG-HRP as secondary antibody was added and incubated at room temperature for 2 h. After the wells were washed, a 1:1 mixture of hydrogen peroxide and tetramethylbenzidine was added and incubated at room temperature for 1 h. The reaction was stopped with 2N H₂SO₄, and the results were read with a microtiter reader (V-max; Molecular Devices Corporation, Menlo Park, Calif.) at a wavelength of 450 nm.

Electron microscopy for the bacterial morphology

Both the wild-type strain and its isogenic mutant were harvested from blood agar plates and gently suspended in phosphate-buffered saline. The negative stain was based on the method of Josenhans *et al*^[7]. The morphology, especially the flagella filaments, was compared between these two strains under the transmission electron microscope (Hitachi, H7000, Tokyo, Japan).

In vitro motility testing and in vivo animal experiments

Both the wild type strain and mutant were used in *in vitro* motility tests based on the method of Kim *et al*^[9]. Besides the *in vitro* motility test, *in vivo* animal experiments were conducted. A total of 90 six- to eight-wk-old male BALB/c mice (Iffa, L'Arbresle, France) were allocated into three study groups, challenged with wild-type *H pylori* (W group, *n* = 30), the *flgK* mutant (M group, *n* = 30), and wild-type strain plus pre-immunization with r-FlgK (I group, *n* = 30). In the I group, mice were immunized ip With 100 μ g of r-FlgK mixed with complete Freund's adjuvant, followed by one further immunization with 50 μ g of r-FlgK with incomplete Freund's adjuvant. These mice were inoculated through gastric gavage with

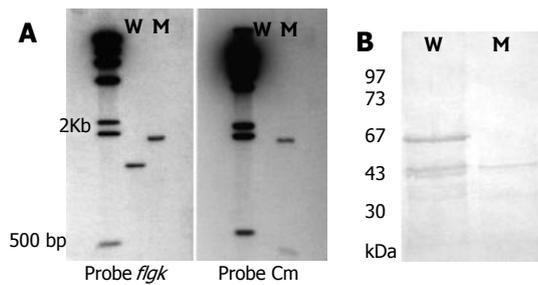


Figure 1 A: Southern blotting of genomic DNA from *H pylori* 250 and its *flgK* mutant digested with *Hind*III. The probes used were specific for the *flgK* gene, chloramphenicol resistance (Cm) gene and λ DNA. Lane W, wild-type strain; lane M, mutant; λ *Hind*III marker used as a molecular size standard; B: Western blotting of anti-r-FlgK polyclonal antibodies to confirm the phenotype of *flgK* isogenic mutant (absence of 67 kDa band). Lane W, wild-type strain. Lane M, mutant. Low molecular weight protein markers.

5×10^8 CFU of wild-type or mutant strains for consecutive 3 d periods^[13]. One week after inoculation, half of the surviving mice were sacrificed by spinal dislocation in each study group. The remaining mice were all sacrificed on the end of 4th wk. Gastrectomy was then performed in each mouse, to be stained with hematoxylin-eosin (HE) for severity of gastric inflammation and Warthin-Starry silver stain for colonization of *H pylori*. These slides were evaluated on a blind-coded basis to assess the total density of *H pylori* colonization (HPD) of each mouse ranging from 0-15^[13]. The serum of each sacrificed mouse was also collected for the serological assay.

Serological response of antibody to FlaA and r-FlgK

This study has prospectively enrolled 70 dyspeptic patients, who have no known history of anti-*H pylori* therapy, to receive panendoscopy and serum samplings. During panendoscopy, the endoscopic diagnosis and the gastric biopsy were taken for the checkup of *H pylori* density (HPD, range 0-15)^[14]. The serological responses of these clinical patients to r-FlgK, and also to the other *H pylori* flagella related proteins like FlaA^[15] were analyzed to test their correlation to the colonization density of *H pylori* in the human stomach. The sera of 70 dyspeptic patients were re-sorted for determination of the antibody titer to r-FlgK and FlaA (HPA 5040, Ibt-Immunological & biochemical test system GmbH, Reutlingen, Germany) by the ELISA method. In addition, the sera of the mice in 3 different study groups were also re-sorted to check the antibody titer to r-FlgK and FlaA.

Statistical analysis

The Student's *t*-test was applied as appropriate for the parametric difference of HPD between mice sacrificed on the 1st wk and on the 4th wk within the same group. The one-way-ANOVA test with Bonferroni's method was used for multiple testing of data, including HPD, titer of anti-r-FlgK, and titer of anti-FlaA in both human and mice settings. All tests of significance were two-tailed with a *P* value < 0.05 taken as significant.

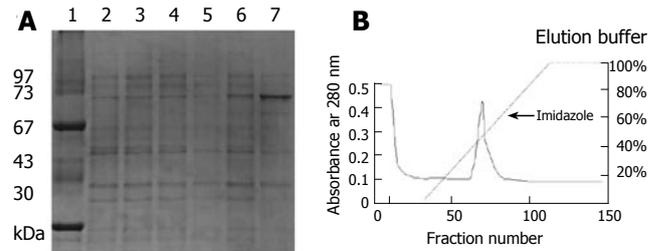


Figure 2 The r-FlgK (73 kDa) can be expressed under IPTG induction by SDS-PAGE. A: Lane 1: Low molecular protein marker, Lane 2: *E. coli* BL21 strain, Lane 3: *E. coli* BL21 strain, IPTG induction, Lane 4: pET-30b/*E. coli* BL21 strain, Lane 5: pET-30b/*E. coli* BL21 strain, IPTG induction, Lane 6: *flgK*/*E. coli* BL21 strain, Lane 7: *flgK*/*E. coli* BL21 strain, IPTG induction; B: The purification of r-FlgK protein by Ni²⁺-chelating chromatography column.

RESULTS

Disruption of the *flgK* gene and cloning of the r-FlgK protein

A pair of primers was used to amplify the *flgK* gene. The PCR product was confirmed by sequencing analysis. The *flaK* gene of *H pylori* hp250 contained 1821 bp, coding for a predicted 606 amino acids, and had 95.7% identity with published sequences^[16]. It has been deposited in GenBank under the accession No. AF333079. Gene replacement was used to disrupt the *flgK* gene. The successful creation of the *flgK* mutant was confirmed with southern blotting (Figure 1A), and western blotting analysis was used to detect the protein phenotype (Figure 1B). The r-FlgK protein was present in a 73 kDa band on the SDS-PAGE gels (Figure 2A). The r-FlgK protein was efficiently eluted from the Ni²⁺-chelating chromatography column by 500 mmol/L imidazole (Figure 2B). Identification of the purified protein of FlgK was confirmed via N-terminal sequence analysis of the protein; the first 10 amino acids (Gly-Gly-Ile-Leu-Ser-Ser-Leu-Asn-Ala-Ser) were confirmed exactly as predicted from the sequence.

Morphological difference under electron microscopy and in vitro motility assay

Under EM, no flagellar filament was observed in the mutant strain (Figure 3B), whereas the polar flagella were observed in wild-type strain (Figure 3A). No motility (indicated by the absence of a halo) was observed in the mutant strain (Figure 3D), whereas the halo was observed in wild-type strain (Figure 3C). Adding a high titer (as high as 1:50) of anti-r-FlgK polyclonal antibody (Figure 3E) had a more evident inhibition of the motility of wild-type *H pylori* than adding a lower titer (1:200) of antibody (Figure 3F).

Colonization assay and serological responses of the in vivo mouse model

All the mice in the three study groups, sacrificed on either the 1st wk or the 4th wk, had *H pylori* colonization. In Table 1, on the 1st wk of the study, the mean HPD of the mice in M group infected by the *flgK* mutant was significantly lower than that of I group, receiving pre-immunization by r-FlgK, and that of W group, infected by the wild-type strain (*P* < 0.05). On the 4th wk, the mean HPD of the mice in the M group remained significantly lower than that

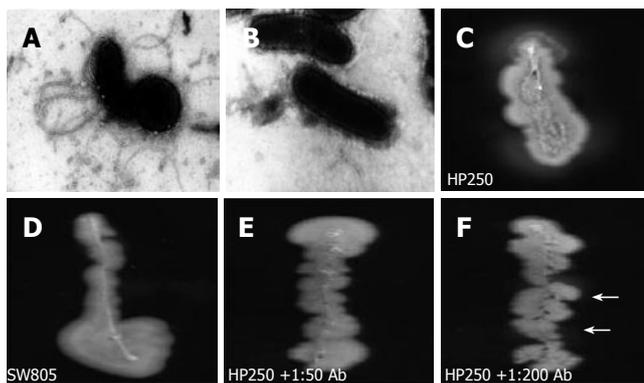


Figure 3 The electron microscopy disclosed wild-type *H. pylori* had full-length sheathed flagella (A), which was not observed in the *flgK* mutant (B). *In vitro* motility testing disclosed halos of wild-type strain (C), whereas the mutant was without halo (D). An evident halo (arrows) indicated a poorer inhibition of the motility of wild-type *H. pylori* by adding 1:200 anti-r-FlgK polyclonal antibody (F) than by adding 1:50 of the antibody (E).

of the W group. The mean HPD of mice in the I group had a significant decrease in those sacrificed on the 4th wk, when compared to those on the 1st wk (3.89 vs 5.24, $P < 0.005$). Moreover, the mean HPD of mice sacrificed on the 4th wk became significantly lower in the I group than in the W group (3.89 vs 6.59, $P < 0.05$).

The serological titers to FlaA, in either 1-wk or 4-wk experiments, were similar among the three mice groups (Table 1). In contrast, the serological titer to r-FlgK of both 1-wk and 4-wk serum samples, were (ranked in an downward order): I group, W group, and then M group ($P < 0.05$).

Serological response to flagella protein in clinical patients

In Table 2, the serological response to FlaA was significantly higher for patients with high *H. pylori* colonization than for patients with low or no *H. pylori* colonization ($P < 0.05$). However, the serologic response to r-FlgK were all less than 0.6 (in titer level) for the three patient groups, and thus were not different between any two patient groups (Table 2).

DISCUSSION

Besides urease production and adhesin, motility enhanced by unipolar flagella is required for *H. pylori* colonization^[5,6]. The motility of *H. pylori* is provided by sheathed flagella, consisting of two flagellins (FlaA and FlaB) encoded by *flaA* and *flaB*^[6,7]. *In vitro* experiments with isogenic *H. pylori* strains of mutated *flaA* and *flaB* confirmed both flagellins are required for motility^[8]. In our study, the isogenic *flgK* mutant of *H. pylori* was disclosed to be without flagella under EM (Figure 3B). *In vitro* experiments revealed the mutant (Figure 3D) had a worse motility than the wild-typed strain (Figure 3C). Our findings confirmed that loss of flagella in *H. pylori* could occur by the disruption of its *flgK*, as well as by other previously reported flagellar genes.

Linkages between flagella and virulence have been observed previously in bacteria other than *H. pylori* isolates. In bacterial species other than *H. pylori*, loss of the ability to produce flagella generally has resulted in a less virulent

Table 1 *H. pylori* gastric colonization and the corresponding serologic responses to FlaA and r-FlgK of *H. pylori* in different mouse groups

Mean (SD)	W group	M group	I group
1-wk mouse groups (n = 15)	(n = 15)	(n = 15)	(n = 15)
Anti-FlaA (A)	0.312 (0.141)	0.301 (0.115)	0.289 (0.128)
Anti-r-FlgK (A) ^{a,b,c}	0.459 (0.066)	0.329 (0.085)	1.258 (0.692)
HPD (0-15) ^{a,c}	5.35 (2.08)	3.65 (2.87)	5.24 (2.15)
4-week mouse groups (n = 13)	(n = 14)	(n = 14)	(n = 14)
Anti-FlaA (A)	0.345 (0.105)	0.324 (0.115)	0.298 (0.101)
Anti-r-FlgK (A) ^{a,b,c}	0.682 (0.395) ¹	0.351 (0.052)	1.325 (0.171)
HPD (0-15) ^{a,b}	6.59 (2.78) ¹	4.25 (2.15)	3.89 (1.85) ¹

W group: mice challenged with wild-type *H. pylori*; M group: mice challenged with isogenic *flgK*-mutant; I group: mice received pre-immunization of r-FlgK before challenge of wild-type *H. pylori*; HPD: total *H. pylori* density of the mice's stomachs by histology. The value of anti-FlaA and anti-r-FlgK was indicated as optical density (A) checked by ELISA with a 1:1000 dilution of serum. ^aindicated significant difference between W and M mice groups; ^bbetween W and I mice groups; ^cbetween I and M mice groups (One way ANOVA test with Bonferroni's method, $P < 0.05$); ¹ indicated significant difference of this parameter between the 1-wk and the 4-wk samples within the same mouse group (Student's *t* test, $P < 0.05$).

Table 2 The serologic responses against FlaA and r-FlgK of *H. pylori* among patients with different gastric colonization

Serological A titer	<i>H. pylori</i> -negative	Low colonization	High colonization
Mean (SD)	(n = 20)	(n = 20)	(n = 30)
Anti-FlaA ^{a,b}	0.268 (0.152)	0.298 (0.118)	0.398 (0.159)
Anti-r-FlgK	0.468 (0.154)	0.501 (0.258)	0.521 (0.233)

Low colonization and high colonization were defined by the total *H. pylori* density in the human stomach scored as less or equal to 5 and more than 5, respectively. A: indicates the optical density of titer checked by ELISA with a 1:1000 dilution of serum. ^aindicated significant difference between patients without *H. pylori* infection and with high colonization; ^bindicated significant difference between patients with low and with high colonization (One way ANOVA test with Bonferroni's method, $P < 0.05$).

organism^[17-19]. With respect to *H. pylori*, nonmotile mutants of *flaA*, *flaB*, or *fljD* genes could decrease motility and thus decrease the ability to establish persistent colonization of *H. pylori* in an *in vivo* mouse model^[6-9]. Such data demonstrated that full motility, mediated by these three flagellar associated genes or others, should be an essential virulence factor of *H. pylori*. These flagellar associated genes are thus possible targets for novel therapeutic or preventive vaccination for *H. pylori* infection. Besides *flaA*, *flaB*, and *fljD*, our study should be the first study to reveal another novel target, *flgK*, as able to regulate the presence of flagella on *H. pylori*.

In Figure 2, the r-FlgK protein of *H. pylori* was purified in this study. The anti-r-FlgK polyclonal antibody was obtained from a rabbit challenged with such purified r-FlgK. The study thus further tested whether anti-r-FlgK polyclonal antibody could inhibit the motility of the wild-type *H. pylori* isolates. *In vitro* motility testing of wild-type *H. pylori* was more inhibited by a 1:50 titer (to give no halo) (Figure 3E) than by a 1:200 titer of anti-r-FlgK polyclonal antibody (which resulted in a faint halo) (Figure 3F).

These findings supported the hypothesis that the immune response to r-FlgK of *H pylori* could possibly interfere with *H pylori* colonization of the infected host.

In Table 1, on the 1st-week study, the mice inoculated with the mutant were found with significantly lower HPD than those inoculated with the wild-type strain (3.65 vs 5.35, $P < 0.05$). The titer of anti-r-FlgK was also lower in the M group than in W group (0.329 vs 0.459, $P < 0.05$). The poor serological response to r-FlgK in the M group indirectly indicated that deficient production of FlgK occurred after destruction of the *flgK* gene. Moreover, deficiency of FlgK in the mutant strain decreased its colonization ability in mice. Such above-mentioned difference was consistently found on the 4th wk (Table 1). On the 4th wk study, the titer of anti-r-FlgK of the immunized group was significantly higher than those of the other two groups (1.325 vs 0.682 and 0.351, $P < 0.05$). On account of this higher titer of anti-r-FlgK antibody (which has the ability to inhibit motility) (Figure 3E and 3F), mice receiving pre-immunization had lower HPD than those mice in W group (3.89 vs 6.59, $P < 0.05$). This evidence was also supported by the limited (< 1.0) titers to r-FlgK of the clinical *H pylori*-infected patients (Table 2). These interesting findings supported the ability of an enhanced immune response to r-FlgK to inhibit the motility of *H pylori*.

The mean titers of anti-FlaA in these three mouse groups were not different on either the 1st or 4th wk study (Table 1). This data suggested the interference with *H pylori* colonization in mice was mainly accounted for by the response to the *flgK* gene product rather than the *flaA* gene.

In contrast to the poor serological responses to anti-r-FlgK, these clinical patients were disclosed to have significant differences in the titer of anti-FlaA Ab among patients with different *H pylori* colonization density (Table 2). This implied the serological response to FlaA could be more sensitive than anti-r-FlgK to predict the presence of *H pylori* infection. However, the titer of anti-FlaA was increased with the increment of *H pylori* colonization (Table 2). The function of anti-FlaA antibody may just serve as a serological marker rather than a promising immune protection against *H pylori* infection.

In summary, FlgK encoded by the *flgK* gene plays an important role in flagella formation and motility of *H pylori*. The deficient FlgK of *flgK*-mutant and the pre-immunization with r-FlgK can effectively decrease *H. pylori* colonization. Therefore, FlgK of *H pylori* may serve as a potential target to inhibit the motility and colonization of *H pylori* in the infected host.

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BASIC RESEARCH

Experimental gastric dysrhythmias and its correlation with *in vivo* gastric muscle contractions

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Abstract

AIM: To study the direct correlation between gastric dysrhythmias and *in vivo* gastric muscle tone.

METHODS: Five healthy dogs were implanted with 4 pairs of electrodes along the greater curvature, with a strain gauge (SG) being sutured parallel to the distal electrodes (2 cm above the pylorus). Intravenous vasopressin was given to induce gastric dysrhythmia. The percentage of regular slow waves and SG energy were calculated.

RESULTS: (1) the regularity of gastric myoelectric activity (GMA) was reduced during and after infusion of vasopressin; (2) SG energy was significantly decreased during the infusion of vasopressin; (3) the decrease in SG energy was well correlated with the reduction in GMA regularity; (4) SG energy was negatively correlated with bradygastria and tachygastria.

CONCLUSION: Vasopressin inhibits gastric contractions and impairs gastric slow waves; gastric dysrhythmias are associated with the reduced antral muscle contractions, and are indicative of antral hypomotility.

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Key words: Gastric myoelectric activity; Bradygastria; Tachygastria; Gastric muscle contraction; Canine study

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INTRODUCTION

Gastric motility is under the control of gastric myoelectric activity (GMA). Normally GMA originates from the junctional area of the fundus and proximal stomach, propagates aborally, controlling the frequency and direction of gastric contractions. Disturbances in GMA, so-called gastric dysrhythmias, have been frequently observed in patients with various diseases including gastroparesis^[1], dyspepsia^[2], anorexia nervosa^[3], gastroesophageal reflux diseases (GERD)^[4], motion sickness^[5,6] and pregnancy^[7], etc. Gastric dysrhythmias have also been linked to gastrointestinal symptoms such as nausea and vomiting^[5,6], and improvement of gastric dysrhythmias seems to be associated with relief of such symptoms^[5,8].

Gastric dysrhythmias are classified into tachygastria (frequency higher than normal), bradygastria (frequency lower than normal) and arrhythmia (no rhythmic activity), based on the dominant frequency of GMA. Although it is believed that gastric motility could be affected inevitably, the relationship between gastric dysrhythmias and gastric motility has not well been established. Various techniques including electrogastrography (EGG), gastric emptying and simultaneous recording of EGG and intraluminal pressure have been applied, and available evidence suggests that tachygastria may be associated with hypogastric motility^[9-12], while the effect of bradygastria on gastric motility remains controversial^[13,14]. The association between specific gastric dysrhythmia and gastric muscle contractions has never been carefully investigated.

To address these issues, in this study we specifically evaluated: (1) the effect of intravenous vasopressin on GMA; (2) the correlation between gastric muscle tone and GMA; (3) the effect of experimentally-induced gastric dysrhythmias on gastric muscle contractions, in a canine model.

MATERIALS AND METHODS

Animal preparations

Seven healthy female hound dogs (15-22 kg) were anesthetized with intravenous infusion of thiopental sodium (20 mg/kg) and maintained with inhalation of isoflurane (1%-2%). A midline laparotomy was performed, and four pairs of temporary cardiac pacing wires (A&E Medical, Farmingdale, NJ) were implanted under the serosal surface along the greater curvature of the stomach. The most distal pair was 2 cm above the pylorus, and the distance between adjacent pairs of electrodes was

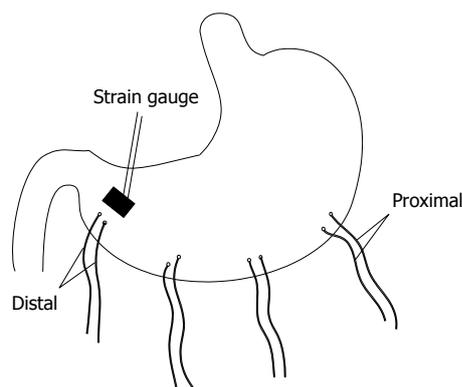


Figure 1 Surgical preparations of stomach. Four pairs of temporary electrodes were implanted along the greater curvature at a distance of 4 cm. The most distal pair was 2 cm above the pylorus. A strain gauge was sutured on the serosal wall parallel to the most distal pair of electrodes.

4 cm (Figure 1). The electrodes in each pair were 1 cm apart. The electrodes were affixed to the gastric serosa by un-absorbable suture in the seromuscular layer of the stomach. The wires were brought out through the anterior abdominal wall, channeled subcutaneously along the right side of the trunk, and placed outside the skin for the attachment for recording gastric myoelectric activity. A strain gauge was sutured on the serosal wall parallel to the most distal pair of electrodes (Figure 1).

The study was initiated about 10 d after the surgery. The protocol was approved by the Animal Use and Care Committee of the University of Texas Medical Branch at Galveston, Texas, USA.

Experimental protocols

Experiments were performed in overnight fasted, fully recovered animals. One study session was performed in each animal. Vasopressin was administered intravenously to induce gastric dysrhythmias in each session. In brief, after a 20 min baseline recording, Vasopressin (0.5 U/kg) was infused continuously for 20 min, followed by another two 20 min recovery periods. GMA and strain gauge signal were recorded simultaneously throughout the session.

Recording of gastric myoelectrical activity and gastric muscle tone

Gastric electrical activity and gastric muscle tone were recorded using a multi-channel recorder system (Acknowledge, Biopac System, Santa Barbara, CA). All signals were displayed on a computer monitor and saved on a hard disk by an HP Pentium IV PC. For GMA, the low and high cutoff frequencies of the amplifier were 0.05 Hz and 35 Hz, and the sampling frequency was 20 Hz. The recordings were then low-pass filtered with a frequency of 1 Hz and re-sampled at a frequency of 24 Hz to reduce the volume of data and potential artifacts before the final review.

Analysis of gastric myoelectrical activity and gastric muscle tone

Regularity of GMA: The percentage of 4- to 6-cpm slow waves is a quantitative assessment of the regularity

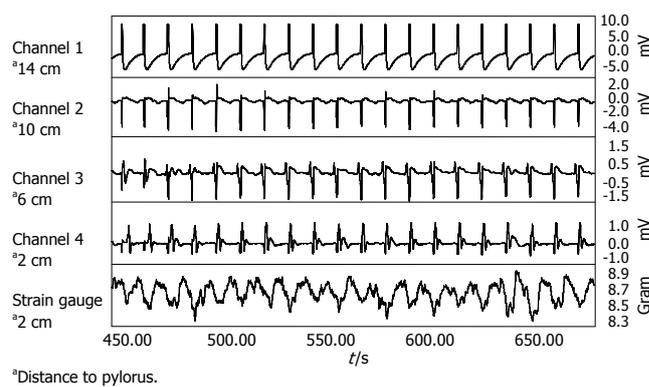


Figure 2 Sample tracings of gastric myoelectric activity recorded from implanted temporary electrodes and gastric contractions from the attached gastric strain gauge. Top 4 channels are gastric myoelectrical activities recorded using serosal electrodes implanted along the greater curvature. The bottom tracing shows gastric contractions measured from a strain gauge implanted in the distal stomach parallel to Channel 4. It was noticed that slow waves propagate from proximal to distal stomach and each gastric slow wave was coupled with a gastric contractile event.

of the GMA measured from the EGG. It was defined as the percentage of time during which normal 4- to 6-cpm gastric slow waves were observed in a specific EGG recording. The percentage of normal 4- to 6-cpm slow waves was computed from the running power spectra of the GMA using an adaptive spectral analysis method^[15]. One power spectrum was generated for every 2 min of EGG data, and the spectral peaks in each spectrum were examined visually. A spectrum was defined as normal if it had a clear peak in the 4- to 6-cpm range. The percentage of regular 4- to 6-cpm slow waves was determined by computing the ratio between the numbers of normal and total spectra.

Gastric dysrhythmias: The percentage of time of gastric dysrhythmias was also computed using running spectral analysis. Bradygastria was defined as a frequency of 0.5-4.0 cpm. Tachygastria was defined as a frequency of 6.0-15.0 cpm. Arrhythmia was defined as any irregular rhythm.

Gastric muscle tone: Reflected by the energy of the strain gauge signals, which were derived by calculating the area under the curve.

Statistical analysis

GMA regularity within each session was compared with One-Way ANOVA. The correlation between gastric muscle tone and gastric dysrhythmias was analyzed with Pearson's correlation test. All data were presented as mean \pm SE. $P < 0.05$ was taken as significance.

RESULTS

Gastric myoelectrical activity and gastric muscle tone at baseline

Intrinsic, distally propagating gastric slow waves were observed in all animals at a mean frequency of 5.6 ± 0.3 cpm, with a range of 4.8-6.2 cpm. Rhythmic variations in gastric muscle tone were consistently present, and coupled with each gastric slow waves at an identical frequency (Figure 2).

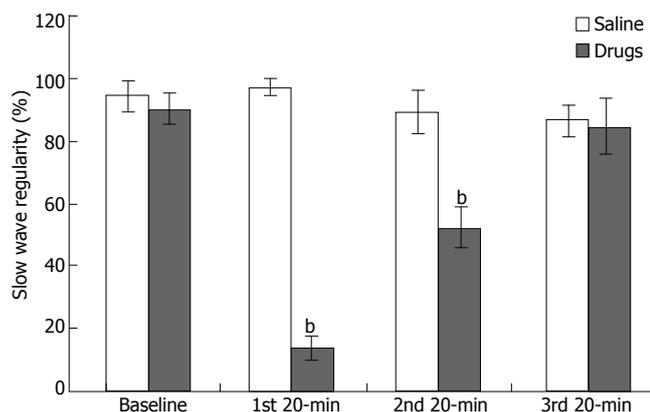


Figure 3 The regularity of gastric myoelectric activity was reduced with and after infusion of vasopressin, and returned somewhat during the last 20-min recovery period. ANOVA, ^b $P < 0.001$ baseline vs 2nd or 3rd 20-min period ($n = 7$).

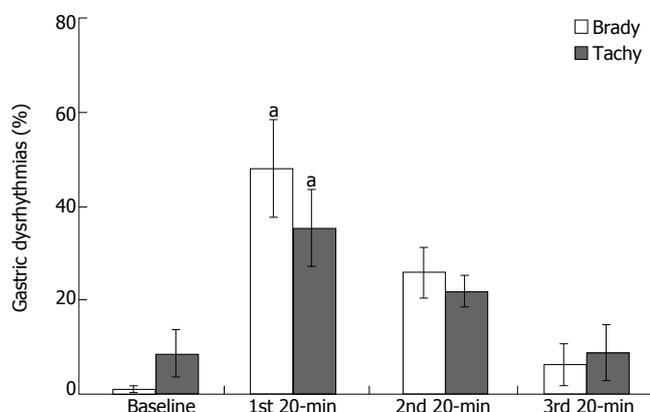


Figure 4 Distribution of gastric dysrhythmias induced by intravenous infusion of vasopressin. Similar percentage of tachygastric and bradygastric was observed, which contributed to the irregularity of gastric myoelectric activity. ANOVA, ^a $P < 0.05$ vs baseline and 3rd 20-min period ($n = 7$).

Effect of vasopressin on GMA

The regularity of GMA was $90.1\% \pm 5.0\%$ at baseline. It was reduced to $13.9\% \pm 3.9\%$ and $52.2\% \pm 6.4\%$ during the following two 20-min periods with and after infusion of vasopressin ($P < 0.01$), and returned to $84.7\% \pm 9.2\%$ during the last 20-min period (Figure 3).

The decrease in the regularity of GMA was attributed to an increase in both bradygastric ($48.0\% \pm 10.4\%$ vs $1.1\% \pm 0.8\%$) and tachygastric ($35.4\% \pm 8.2\%$ vs $8.7\% \pm 5.0\%$) (Figure 4).

Effect of vasopressin on strain gauge energy

The strain gauge energy was significantly decreased during the 20-min infusion of vasopressin. The total energy was 48.4 ± 1.3 dB at baseline, and decreased to 36.6 ± 4.5 dB, 44.5 ± 4.3 dB, 45.8 ± 1.9 dB during the following consecutive three 20-min periods during and after the infusion (vs baseline, $P < 0.05$).

Correlation of GMA and strain gauge energy

The decrease in strain gauge energy was positively correlated with the decrease of GMA regularity ($r = 0.96$, $P < 0.05$). Gastric muscle contractions were reduced or disappeared during periods of bradygastric or tachygastric

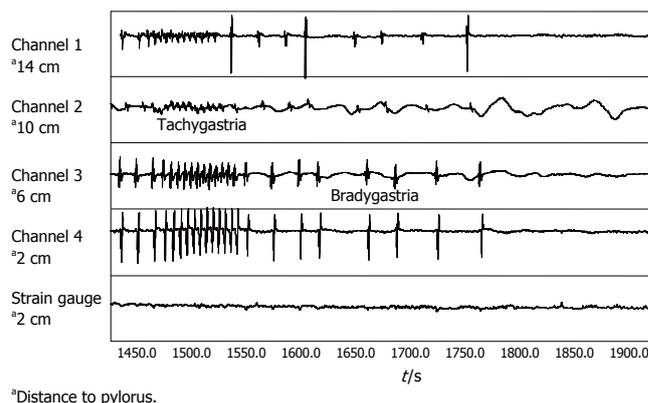


Figure 5 Illustration of gastric dysrhythmias induced by intravenous Vasopressin. Top 4 channels are gastric myoelectrical activities recorded using serosal electrodes implanted along the greater curvature. The bottom tracing shows gastric contractions measured from a strain gauge implanted in the distal stomach parallel to Channel 4. It was noticed that gastric muscle contractions were absent during periods of tachygastric and bradygastric.

(Figure 5). Strain gauge energy was negatively correlated with bradygastric ($r = -0.96$, $P < 0.05$) and tachygastric ($r = -0.95$, $P > 0.05$).

DISCUSSION

Through this study we have found: (1) Intravenous vasopressin at proper doses consistently produced gastric dysrhythmias; (2) strain gauge implanted on gastric wall could reliably detect gastric muscle contractions; gastric muscle contractions were coupled with intrinsic gastric slow waves, and each gastric slow wave was capable of inducing a change in gastric muscle tone; (3) gastric muscle contractions were positively correlated with the regularity of the gastric slow waves; and (4) the reduction or disappearance in gastric muscle contractions was associated with both bradygastric and tachygastric.

Considering the fact that all muscle contractions are coupled and superimposed with myoelectric activities, it is strongly believed that gastric dysrhythmias could cause disturbances in gastric motor functions^[16]. A larger number of clinical studies have been performed recently, and indeed, gastric dysrhythmias have been observed in various motility disorders like gastroparesis, functional dyspepsia and motion sickness, *etc*^[1-7]. In patients with gastroparesis, gastric emptying scintigraphy and EGG were performed concurrently, and it was found that postprandial gastric dysrhythmias correlated with delays in solid phase gastric emptying^[17]. Gastric dysrhythmias may also be in association with the occurrence of GI symptoms^[5,6]. We have noted on many occasions that degeneration of GMA preceded the occurrence of nausea and vomiting in our previous canine studies. Other investigators also reported similar findings^[5,6,18], and all these indicate a possible causative role for the dysrhythmia in the production of nausea and vomiting.

Only few studies have been conducted to qualitatively assess the association between gastric dysrhythmias and gastric motility and there have been no quantitative studies. The first report on tachygastric in humans was published by Telander *et al*^[11]. They reported a 5 month-

old male infant suffering from severe gastric retention and symptoms of intractable nausea, vomiting and weight loss. The symptoms were attributed to impaired motor function of the stomach. The patient underwent resection of the distal $\frac{3}{4}$ of the stomach. The excised tissues were studied *in vitro* by means of intracellular electrodes, and abnormally fast slow waves (> 5 cpm) were detected^[11]. The association between gastric dysrhythmia and gastric motor disorders was further substantiated by other studies. You *et al*^[12] observed tachygastria in a 26-year-old woman with persistent nausea, vomiting and abdominal pain who was found to have severe impairment of antral motor functions. In contrast to tachygastria, data on bradygastria are inconsistent. Van Der Schee *et al* observed bradygastria in dogs and found that it was correlated with strong antral contractions^[13], while Abell *et al*^[14] induced bradygastria in humans with glucagon, and found that it was associated with absence of antral contraction.

In our present study, we attached a strain gauge to the site of recording electrodes, which allows us to study the direct correlation of gastric contractions and GMA. In comparison to the traditional intraluminal manometry, strain gauge is more sensitive and more direct in the detection of subtle contractions of gastric wall than any other methods. In addition, gastric dysrhythmias in this study were assessed quantitatively rather than qualitatively in the previous studies. Our results indicate that both tachygastria and bradygastria are negatively associated with gastric muscle contractile activity, which is consistent with most of the previous report^[11-14].

The exact mechanisms causing gastric dysrhythmias have not been fully understood, though it is well known that GMA is generated by interstitial cells of Cajal, and is influenced by central and autonomic nerve systems, certain hormones and peptides. Tachygastria and bradygastria are different. Tachygastria usually originates from distal stomach, and can be considered as an ectopic rhythm, while transient or persistent bradygastria usually originates from the region of the normal pacemaker at a reduced frequency. Gastric dysrhythmia can be induced experimentally with various agents including vasopressin^[14,19-21]. Vasopressin is a peptide released into the peripheral circulation from the pituitary during experimental motion sickness, and has been confirmed to induce gastric and intestinal dysrhythmia and symptoms of nausea and vomiting^[20-22]. An elevated plasma level of vasopressin was reported in postsurgical patients with impaired gastrointestinal motility^[23]. Vasopressin was known to reduce mesenteric blood flow, which was frequently used in patients with esophageal variceal bleeding^[24], and this vasoconstrictive effect might be the mechanism involved in the induction of GI dysrhythmias.

Gastric myoelectric activity can be recorded in several ways including intraluminal electrodes, surface electrogastrogram and serosal electrodes. Placement of intraluminal electrodes is invasive and uncomfortable. It is very difficult to direct the electrodes to specific sites of stomach and electrodes can be easily dislodged. EGG is a convenient summation technique with various disadvantages: (1) derived data on frequency and power are not strictly region specific; (2) susceptible to a variety

of artifacts; (3) cannot discriminate chaotic rhythm from uncoupling; and (4) susceptible to myoelectric interference from other gut organs, and cutaneously acquired signals may not be gastric in origin^[25]. Serosal recordings, on the other hand, do not have these potential technical problems, and is the most reliable *in vivo* GMA recording except for the need for surgery. This method has allowed us to put the recording electrodes and strain gauge at the same location, thus providing an excellent tool for the study of the direct correlation of dysrhythmias and gastric contraction, which has never been done before.

Another interesting finding from this study is that under control conditions each gastric slow wave seems capable of inducing a certain level of mechanical contractions in gastric muscle. These contractions are so subtle that they could only be reflected by strain gauge, other than other techniques, including intraluminal manometry. This observation is inconsistent with traditional view that only gastric slow waves superimposed with spike activities are capable of inducing gastric contractions.

In summary, in this study we have observed that intravenous administration of vasopressin could impair gastric slow waves and gastric muscle contractions. Experimentally induced tachygastria and bradygastria are negatively correlated with the gastric muscle contractions, suggesting that gastric dysrhythmias, either tachygastria or bradygastria, may be indicative of gastric hypomotility.

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Caerulein-induced pancreatitis in rats: Histological and genetic expression changes from acute phase to recuperation

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Abstract

AIM: To study the histological and pancreatitis-associated protein mRNA accumulation changes of pancreas from acute phase of caerulein-induced pancreatitis to recuperation in rats.

METHODS: Acute pancreatitis was induced by caerulein in male Wistar rats and followed up for 90 d by histological and mRNA analyses of pancreas. Pancreases were dissected at 0, 9, 24 h and 3, 5, 15, 30, 60, 90 d post-induction. Edema (E), polymorphonuclear neutrophil (PMN) infiltration, cytoplasmic vacuolization (V), zymogen granule depletion (ZD) and acinar disorganization (AD) were microscopically evaluated. Accumulation of pancreatitis-associated protein (PAP) and L13A mRNAs were quantified by real-time PCR.

RESULTS: The main histological changes appeared at 9 h post-induction for PMN infiltration and cytoplasmic V, while at 24 h and 3 d for E and ZD, respectively. All the parameters were recovered after 5 d, except for ZD which delayed more than 30 d. The main AD was observed after 15 d and values returned to normal after 30 d. Similarly to histological changes, accumulation of the PAP mRNA was increased at 9 h with the highest accumulation at 24 h and differences disappeared after 5 d.

CONCLUSION: From the acute phase to recuperation of pancreatitis, regeneration and re-differentiation of pancreas occur and PAP expression is exclusively an acute response of pancreatitis.

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Key words: Acute pancreatitis; Histological changes; PAP

mRNA; L13A mRNA

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INTRODUCTION

Acute pancreatitis (AP) is an inflammatory disease of the pancreas associated with autodigestion of the gland, as a consequence of the intrapancreatic activation and releasing of digestive enzymes^[1]. The pathogenic mechanisms involved in the development of acute pancreatitis are not well understood^[2,3] although many studies on it are available. The acute period of pancreatitis is characterized by exocrine insufficiency as a result of important morphological alterations and changes on the expression of a number of genes in the pancreas^[4,5]. If pancreatitis is not fatal, a stepwise regeneration of the morphology is followed^[6].

Some events that regulate the severity of acute pancreatitis are known, involving common inflammatory and repair pathways^[7,8]. Recently it was demonstrated that pancreatitis-associated protein (PAP), an acute phase protein, is itself an important determinant of disease severity^[9]. The expression of this gene is low in the normal pancreas and becomes strongly augmentation after even mild pancreatic inflammation^[10]. This augmentation is assumed in AP induced by caerulein, although it can be assayed in AP induced by retrograde injection of sodium taurocholate. Currently, pancreatitis is induced by supramaximal dose of caerulein and there is no report yet on histological and PAP mRNA changes in the pancreas as response of AP induction.

Therefore, the objective of this study was to evaluate the morphological and PAP mRNA changes in the acute and adaptive phases of pancreatitis induced by caerulein in rats. Morphological changes were evaluated by light microscopy and PAP mRNA levels by real-time PCR.

MATERIALS AND METHODS

Bioassay

The experiments were carried out on 33 male Wistar rats (mean weight 100.8 g and 4-wk old). The animals were fed with standard laboratory chow and water *ad libitum*

under controlled room conditions. Acute pancreatitis was induced according to the method of Dusseti *et al*^[11]. The rats were injected intraperitoneally with caerulein (Sigma Chemical Co., St. Louis, MO, USA) at a dose of 50 µg/kg of body weight per hour for 6 h. The rats were sacrificed at h 9 and 24, and on d 3, 5, 15, 30, 60 and 90 respectively. Three control rats without caerulein injection were sacrificed at h 0 and on d 30 and 90 and dissected. Prior to sacrifice, the rats were anesthetized with 10-15 mg of tiletamine chlorohydrate and zolazepam chlorohydrate (Zoletil 50[®]) per kg of body weight. Pancreas was dissected for histological or RNA analysis. Feeding protocol and animal handling were approved by the local ethics committee.

Histological examination

Pancreatic sections were fixed in 10% formaldehyde, embedded in paraffin, cut (3-5 µm in thickness) with a semi-motorized rotary microtome (Leica RM-2145) and mounted onto slides. The sections were processed for H&E staining and examined by conventional light microscopy (Olympus microscope B × 50) with 20 ×, 40 × and 100 × objectives. Eight random images by objective were recorded by microscope video camera. A pathologist who was blinded to the treatment protocol scored the tissues according to Kyogoku *et al*^[12] for zymogen granule depletion (0, absent; 1, less than 20%; 2, 20%-50%; 3, more than 50%), interstitial edema (0, absent; 1, expanded interlobular septa; 2, expanded intralobular septa; 3, separated individual acini), polymorphonuclear neutrophil (PMN) infiltration (0, absent; 1, less than 20 PMNs per intermediate power field (IPF); 2, 20-50 PMNs per IPF and 3, more than 50 PMNs per IPF), grade of vacuolization based on the percentage of acinar cells with cytoplasmic vacuoles per IPF (0, absent; 1, less than 20%; 2, 20%-50%; 3, more than 50%) and acinar disorganization based on the percentage of area without normal acinar distribution (0, absent; 1, less than 20%; 2, 20%-50%; 3, more than 50%).

RNA preparation

Extracted pancreas was immediately rinsed with RNase-free water (treated with 0.05% diethyl pyrocarbonate, DEPC), homogenized in 1 mL of TRIzol[®] reagent (GIBCO-BRL, Grand Island, NY, USA), frozen and stored at -70°C. Total RNA was extracted by phenol/chloroform extraction and isopropanol precipitation^[13]. Accumulation of PAP mRNA (GenBank access No. NM_053289.1) was analyzed as indicator of pancreatic injury. L13A mRNA (GenBank access No. X68282) was also analyzed as house-keeping gene presumed to be invariant^[14]. All the primers were designed with Primers3 software and synthesized by Sigma Genosys (Woodlands, TX, USA). Their sequences and PCR product sizes are listed in Table 1. Prior to the reverse transcription reaction, potentially contaminating residual genomic DNA was eliminated with DNase I (Invitrogen, Carlsbad, CA, USA) followed by reverse-transcribed using specific primers and SYBR Green RT-PCR reagents (PE Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Synthesized complementary DNA (cDNA) corresponding to 100 ng total RNA was used for real-time PCR.

Table 1 Oligonucleotide primer pairs used for real-time quantitative PCR, binding site and expected size

Primer	Sequence	GenBank No.	Product size (bp)
PAPfw	5' TGAATTATGTCAACTGGGAGAGG 3'	NM-0532891	318
PAPrv	5' TTACTGCTTTCGAAGACATGAGG 3'		
rL13A/Fw	5' AAGCAGGTACTGCTGGG 3'	X68282	261
rL13A/Rv	5' CCAACACCTTGAGGCGTT 3'		

Standard synthesis

Standards for the absolute quantification for PAP and L13A mRNAs were synthesized by Titan one Tube RT-PCR system (Roche Biochemicals, Indianapolis, IN, USA) with the same PCR parameters described above, according to the manufacturer's instructions. Then, PCR products were purified with Wizard[®] SV gel and PCR clean-up system (Promega, Madison, WI, USA) and quantified by fluorometry.

Quantitative PCR analysis

Real-time quantification of PAP and L13A mRNAs was performed with an iCycler iQ detection system (BIO-RAD, Hercules, CA, USA). SYBR Green I assay was used for quantification of all specific genes. For PAP mRNA quantification of each 50 µL SYBR Green PCR, 9.0 µL cDNA (corresponding to 100 ng total RNA), 1.8 µL sense primer (10 µmol/L), 1.8 µL antisense primer (10 µmol/L), 25 µL SYBR Green PCR Master Mix (PE Applied Biosystems) and 12.4 µL PCR-grade water were mixed together. The PCR conditions were 1 cycle at 94°C for 4 min, then 35 cycles at 94°C for 30 s, at 61°C for 30 s and at 70°C for 30 s, with a single fluorescence measurement at the end of the 70°C for 30 s. For L13A mRNA quantification of each 50 µL SYBR Green PCR, 9.0 µL cDNA (corresponding to 100 ng total RNA), 1.2 µL sense primer (10 µmol/L), 1.2 µL anti-sense primer (10 µmol/L), 25 µL SYBR Green PCR Master Mix (PE Applied Biosystems) and 13.6 µL PCR-grade water were mixed together. The PCR conditions were 1 cycle at 94°C for 3 min, then 38 cycles at 94°C for 45 s, at 55°C for 45 s and at 72°C for 1 min, with a single fluorescence measurement at the end of the 72°C for 30 s. In both cases, a melting curve program (60-95°C with a heating rate of 0.5°C for 30 s and continuous fluorescence measurement) and a cooling step to 4°C were added.

Analysis and normalization of real-time PCR data

Optical data obtained by real-time PCR were analyzed by using the default and variable parameters available in the software provided with the iCycler iQ optical system (BIO-RAD, Hercules, CA, USA). The PCR threshold cycle number (CT) for each cDNA standard and cDNA sample was calculated at the point where the fluorescence exceeded the threshold limit. The threshold limit was fixed along the linear logarithmic phase of the fluorescence curves at 10-20 SDs above the average background fluorescence. The number of amplicon cDNA copies was expressed relative to the amount of total cDNA present (ng). Although it might be difficult to determine the absolute amount of a

Table 2 Histological changes in the pancreas of rats with caerulein-induced acute pancreatitis (mean \pm SE)

Period	Zymogen granule depletion	Edema	PMN infiltration	Vacuolization	Acinar disorganization
0 h	0.25 \pm 0.23 ^{ab}	1.25 \pm 0.31 ^{ab}	0.00 \pm 0.14 ^a	0.25 \pm 0.26 ^a	0.5 \pm 0.36 ^a
9 h	1.00 \pm 0.23 ^{bc}	2.25 \pm 0.31 ^{bc}	0.75 \pm 0.14 ^b	2.75 \pm 0.26 ^c	0.75 \pm 0.36 ^{ab}
24 h	1.40 \pm 0.20 ^c	3.00 \pm 0.28 ^c	0.60 \pm 0.12 ^b	2.00 \pm 0.24 ^b	1.00 \pm 0.32 ^{ab}
3 d	3.00 \pm 0.18 ^e	2.17 \pm 0.25 ^{bc}	0.67 \pm 0.11 ^b	2.00 \pm 0.22 ^b	1.33 \pm 0.29 ^{ab}
5 d	2.00 \pm 0.23 ^{cd}	1.25 \pm 0.31 ^{ab}	0.00 \pm 0.14 ^a	1.00 \pm 0.26 ^{ab}	0.75 \pm 0.36 ^{ab}
15 d	2.57 \pm 0.17 ^{de}	0.86 \pm 0.23 ^a	0.00 \pm 0.10 ^a	0.00 \pm 0.20 ^a	2.14 \pm 0.28 ^b
30 d	1.60 \pm 0.20 ^c	0.60 \pm 0.83 ^a	0.00 \pm 0.12 ^a	0.00 \pm 0.24 ^a	0.80 \pm 0.33 ^{ab}
60 d	0.00 \pm 0.19 ^a	0.83 \pm 0.25 ^a	0.00 \pm 0.11 ^a	0.17 \pm 0.22 ^a	0.66 \pm 0.29 ^a
90 d	0.25 \pm 0.13 ^{ab}	0.42 \pm 0.18 ^a	0.00 \pm 0.07 ^a	0.00 \pm 0.15 ^a	0.92 \pm 0.21 ^{ab}

Different letters within a same column differ significantly ($P < 0.05$).

cDNA present in different samples, quantification of test mRNA transcripts was normalized to a reference gene, the L13A gene.

Statistic analysis

Histological data were expressed as mean \pm SE of the scores assigned by the pathologist and evaluated by ANOVA. Also, relation between PAP and L13A after absolute quantification was expressed as mean \pm SE and compared using ANOVA test. $P < 0.05$ was considered statistically significant. NCSS 2001 statistical program was used.

RESULTS

Histological examination

At the beginning (0 h), the architecture of pancreas was normal (Figure 1) with acinar cells exhibiting the typical epithelial polarity. Basal portion of the cells contained their nuclei and apical portion of the secretory vesicles and zymogen granules. Acinar cells in AP displayed moderate vacuolization and light inflammatory infiltration of neutrophils, with the highest score after 9 h post-induction. After 5 d, levels returned to the normal scores maintained to the end of the study. Edema was increased 9 h post-induction with the highest score at 24 h and returned to the normal level on d 5. Zymogen granule depletion was most pronounced on d 3 with later recovery. However, an additional depletion occurred on d 15, returned to the normal levels 30 d post-induction (Table 2). Although some degree of acinar disorganization was observed in the pancreas 9 h after induction, a significant increase was observed on d 15, and returned to the normal level 30 d post-induction (Table 2).

Specific amplification

Melting curve analysis demonstrated that each of the primer pairs (Table 1) amplified a single predominant product with a distinct melting temperature (T_m) as shown for L13A and PAP cDNA in Figure 2. The T_m of products could be seen clearly as a peak in a first derivative plot. The rapid fall of T_m at 87.5°C and 82.0°C for L13A and PAP cDNA respectively indicated the presence of a specific product melting at this temperature.

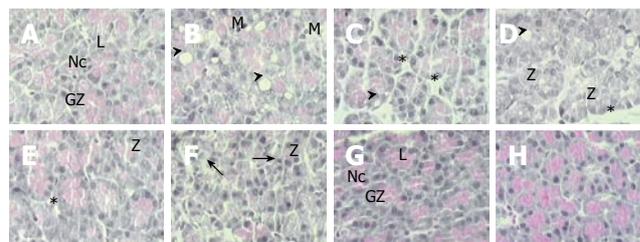


Figure 1 Light-microscopic sections of pancreas (X 133) from 0 h (A) and rats with caerulein-induced acute pancreatitis at 9 h (B), 24 h (C), 3 d (D), 5 d (E), 15 d (F), 30 d (G) and 60 d (H). Normal pancreatic acinus (0 h) shows zymogen granules (GZ) concentrated in the apical pole of the cell close to the acinar lumen (L). The basal region of the cell contains nucleus (N). In AP, PMN infiltration (M), vacuoles (arrowhead), edema (star), zymogen granule depletion (z) and acinar disorganization (arrow) were observed on d 15. On d 30, pancreatic acinus appearance was almost indistinguishable from that at 0 h.

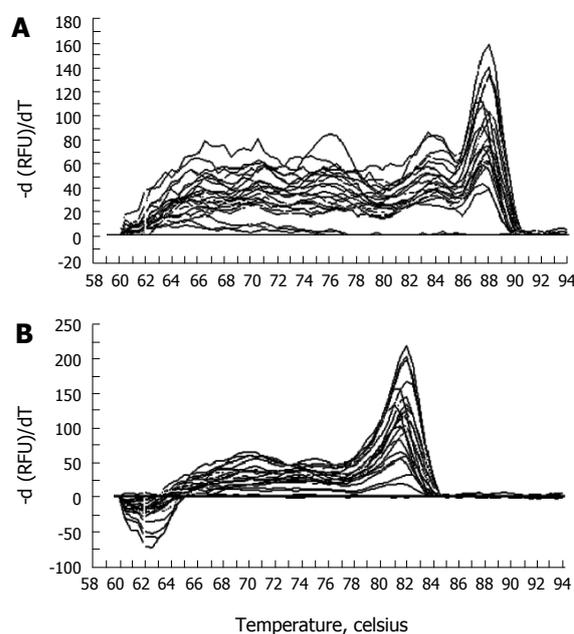


Figure 2 DNA melting curve analysis of amplification products for PAP mRNA (A) and L13A (B).

Absolute detection and quantification

The absolute quantification was estimated from real-time PCR analyses using a standard curve of tenfold serial dilutions ranging from 1.78×10^9 copies to 1.78×10^5 copies of L13A mRNA and 1.46×10^9 copies to 1.46×10^5 copies of PAP mRNA, in 50 μ L of volume reaction. As shown in Figure 3, the mRNA of PAP was detected in basal levels without caerulein (0 h). However, after induction of AP, the PAP mRNA expression was slightly augmented after 9 h, and 3.8-fold the basal level at 24 h. After 5 d, differences disappeared and the accumulation of PAP mRNA was decreased gradually to the basal level.

DISCUSSION

The acute phase of pancreatitis is characterized by a pattern of changes in the morphology of exocrine pancreas, expression of secretory proteins and mRNA levels of different genes^[4,6,15,16]. The observed changes in our study resemble a mild form of AP, characterized by edematous fluid in the extra-cellular space, causing separation of lobules

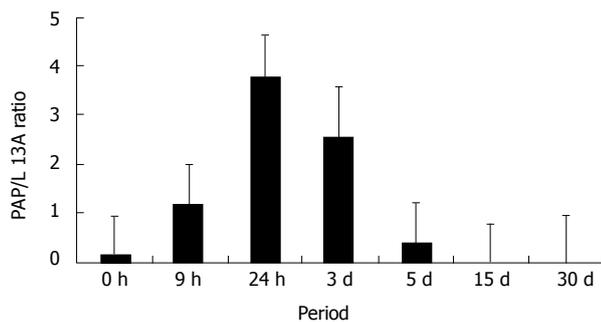


Figure 3 Relative quantification of PAP mRNA in caerulein-induced pancreatitis. Absolute quantification of PAP mRNA as copies was normalized to copies of L13A as determined from standard curves. All data are mean \pm SE.

and acini^[6]. Usually there is mild invasion by neutrophils, the acinar cells lose their zymogen granules, enlarging the acinar lumen. However, few studies^[17-19] have analyzed the morphological changes after the acute phase of pancreatic response.

After acute response, re-differentiation of acinar cells and transition begin with regular acinar cell replete with dense zymogen granules beside cells with a smaller quantity of zymogen granules, on the same microscopic preparation. Intermediate cells change the typical architecture of the pancreas due to the loss of zymogen granules in a particular section. Additionally, the rough endoplasmic reticulum might be reduced and/or rearranged^[6]. In this study, zymogen granule depletion and increase of acinar disorganization were presented after the acute phase of pancreatitis (determined by maximal PAP synthesis and its mRNA accumulation according to Iovanna *et al*^[4] and our results, respectively). These changes are explained in the context of reconstitution of the normal morphology and function of the pancreas.

De Lisle and Logsdon^[20] have shown the differentiation and re-differentiation of mouse pancreatic acinar cells. They immunolabeled acinar cells with monoclonal antibodies specific for acinar or duct cells and showed that more than 97% of the labeled area is acinar positive on d 3, which decreases to approximately 16% on d 9, and then returns to over 97% by day 21 of culture^[20]. Lechene de la Porte *et al*^[21] induced pancreatitis by the injection of trypsin in multiple sites of the rat pancreas and reported that pancreatic repair involves proliferation of cells from intact acini and tubular complexes. This process of differentiation and re-differentiation of pancreatic acinar cells explains our finding of the highest score for acinar disorganization on d 3 and 15 as well as complete recovering of zymogen granules 30 d post-induction.

In pancreatitis not only morphological changes were observed, but also molecular modifications occurred. Iovanna *et al*^[4] showed that PAP is significantly increased 48 h after induction of pancreatitis by retrograde injection of sodium taurocholate in rats, compared to normal pancreas. In spite of methodological differences in the quantified PAP, our results agree with those of Iovanna *et al*^[4]. However, PAP mRNA accumulation is quite differ-

ent between both studies. Differences could be attributable to the pancreatitis induction method, the severity of the problem, as well as the quantification method itself. In our study, after absolute quantification, values were normalized in respect to a housekeeping gene (L13A). Therefore, quantification by real-time PCR may better reflect the actual physiological changes.

Histology of pancreas showed a mild edematous pancreatitis induced by caerulein and the acute response was followed by recuperation process after 15 d of induction, according to acinar disorganization and zymogen granule depletion scores. Although PAP is related to the severity of pancreatitis, its function *in vivo* is not well established. Our findings support that PAP is a protein of acute response, because changes in levels are not observed in reconstitution of the pancreatic normal morphology. In addition, the PAP mRNA changes in AP induced by caerulein, measured by a most sensible technique of quantification as real-time PCR, are similar to those from AP induced by sodium taurocholate.

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BASIC RESEARCH

Inhibitory effects of antisense phosphorothioate oligodeoxynucleotides on pancreatic cancer cell Bxpc-3 telomerase activity and cell growth *in vitro*

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from 72.7% to 51.0%, and a sub-G1 stage cell apoptosis peak appeared in front of G1 stage.

CONCLUSION: Telomerase antisense oligodeoxynucleotide can inhibit the proliferation of pancreatic cancer cell line Bxpc-3 and decrease the telomerase activity and increase cell apoptosis rate *in vitro*.

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Key words: Antisense oligodeoxynucleotide; hTERT; Telomerase; Telomerase reverse transcriptase

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<http://www.wjgnet.com/1007-9327/12/4004.asp>

Abstract

AIM: To investigate the effect of telomerase hTERT gene antisense oligonucleotide (hTERT-ASO) on proliferation and telomerase activity of pancreatic cancer cell line Bxpc-3.

METHODS: MTT assay was used to detect the effect of different doses of hTERT-ASO on proliferation of Bxpc-3 cell for different times. To study the anti-tumor activity, the cells were divided into three groups: Control group (pancreatic cancer cell Bxpc-3); antisense oligonucleotide (hTERT-ASO) group; and nonsense oligonucleotide group decorated with phosphorothioate. Telomerase activity was detected using TRAP-PCR-ELISA. Cell DNA distribution was examined using flow cytometry assay. Cell apoptosis was observed by transmission electron microscope in each group.

RESULTS: After treatment with 6 mmol/L hTERT-ASO, cell proliferation was inhibited in dose- and time-dependent manner. The telomerase activity decreased after treatment with hTERT-ASO for 72 h. Flow cytometry showed the cell number of G₀/G₁ phase increased from 2.7% to 14.7%, the cell number of S phase decreased

INTRODUCTION

Telomerase is a ribonucleoprotein polymerase which adds telomeric sequences onto the ends of chromosomes to compensate for DNA end replication^[1]. Telomerase plays an important role in the development of cellular immortality and oncogenesis^[2]. It is different from reverse transcriptase DNA polymerase of commonly pure proteins. The activated telomerase takes the 3' distal end of telomeres as the primer and its RNA component acts as the template^[3]. In human, telomerase activity has been found in embryonic development, adult germ-line tissues, immortal cell^[4,5] and most malignant tumors^[6,7], while there is no evident expression in normal human tissue other than in germ cell, hemopoietic stem cells and cuticle basal cells^[8,9]. Human telomerase is a ribonucleo-protein complex, composed of a catalytic reverse transcriptase subunit (hTERT), an RNA component (hTR) that serves as a template for the synthesis of telomeric repeats, and an associated protein subunit (TP1)^[10-12]. Previous studies have shown that telomerase activity is found in 85%-90% of all human tumors, but not in their adjacent normal cells^[13,14]. This makes telomerase a good target not only for cancer diagnosis, but also for the development of novel therapeutic agents^[15,16]. Among them, only the expression

of hTERT mRNA is correlated with telomerase^[17], and mainly regulates the expression of human telomerase enzymatic activity^[18-20]. hTERT is a useful marker for telomerase activation^[21,22]. It is associated with the majority of human malignant cancers. Therefore, telomerase should be the target of anti-tumor drugs research.

In this study, we aimed to investigate the inhibitory effects of antisense phosphorothioate oligodeoxynucleotides on human pancreatic cancer cell line Bxpc-3 telomerase activity and cell growth, thereby exploring the potential value of telomerase hTERT gene as a target for antisense gene therapy in pancreatic cancer.

MATERIALS AND METHODS

Cell culture

Human pancreatic carcinoma cell line (Bxpc-3) was obtained from Chinese National Cancer Institute, Chinese Academy of Medical Sciences, Beijing. Cells were cultured in RPMI 1640 medium supplemented with heat-inactivated fetal bovine serum, 2 mmol/L L-glutamine, 100 kU/L penicillin and 100 mg/L streptomycin at 37°C in a humidified CO₂ incubator containing 50 mL/L CO₂ and 950 mL/L air. Cells were counted using 5 g/L trypan blue staining.

Oligodeoxynucleotides synthesis

Oligodeoxynucleotides synthesis was designed as described in GenBank, and they have not the same source searched with computer. Antisense oligodeoxynucleotides (ANS-ODN) with sequence 5'-CTCAGTTAGGGTTAGACA-3', which can recognize the RNA template region of telomerase, and nosense oligodeoxynucleotides (NNS-ODN) with sequence 5'-CATTTCTTGCTCTCCACG-3' were prepared on the 391 DNA synthesizer, and synthesized by Huamei Engineering Company of Beijing. The synthesized oligodeoxynucleotides were subjected to polyacrylamide gel electrophoresis and purified by running at 300 V for 1.5 h.

MTT assay

Cells were seeded at 2×10^6 cells /well in a 96-well plate, 100 μ L per well in three wells. The ANS-ODN and NS-ODN were added to the cultured cells with the final concentrations of 1, 2, 4, 6 μ mol/L, and further cultured for 24, 48, 72, 96 h, respectively. Four hours before the end of culture, 50 μ L (1 g/L) 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, Sigma) was added, which was used to assay the activity of mitochondrial dehydrogenases. Four hours later, DMSO was added to solubilize the crystal product. The plate was then incubated at 37°C for another 12 h. The absorbance at 630 nm was measured with a model 550 microplate reader (Bio-Rad). The percent of cell growth inhibition was calculated as follows: $(A_c - A_e) / A_c \times 100\%$, where A_c is the absorbance value for the controls and A_e for the experimental cells.

Flow cytometry analysis of cell cycle and apoptosis

Approximately 2×10^6 cells were harvested and washed twice with PBS, fixed with 700 mL/L alcohol for 24 h, prepared as single-cell suspension and stored at 4°C,

thawed rapidly at room temperature and centrifuged to collect the cells. The cells were resuspended in a solution containing 10 g/L RNase A (833.5 μ kat/g, 50 mg/L) and 20 mg/L propidium iodide (PI) for 10 min. The cell cycle distribution and apoptosis were determined by the fluorescence of individual cells measured with flow cytometry^[23]. All data were analyzed using Cell Quest software.

Telomerase activity assay

Polymerase chain reaction enzyme-linked immunosorbent assay (PCR-ELISA) was performed following the manufacturer's instructions (Huamei Engineering Company of Beijing). Briefly, 2×10^6 cells were isolated, mixed with 50 μ L lysis buffer and incubated on ice for 30 min. Supernatant was collected after centrifugation at 14 000 g for 20 min at 4°C. In an Eppendorf tube, 2 μ L of the supernatant, 45 μ L of reaction mixture and 30 μ L of liquid olefin were added, and centrifuged for several seconds, placed in the water-bath at 25°C for 30 min, and then subjected to PCR amplification. The PCR conditions were as follows: primer elongation at 94°C for 2 min; telomerase inactivation at 94°C for 30 s; and 35 amplification cycles, each cycle consisted of denaturation at 48°C for 30 s, primer annealing at 72°C for 90 s, and extension at 72°C for 5 min. Then 50 μ L of amplified product and 100 μ L of hybridization reagent solution B were added and mixed; 25 μ L of the mixture was distributed in the wells of a microtitering plate incubated at 37°C for 60 min. The mixture was washed at 37°C for 15 min using 100 μ L of the hybridization reagent solution C, followed by addition of 25 μ L of solution A and B in microtitering and incubation at 37°C for 15 min. Finally, 2 μ L of stop reagent was added. The value in each well was read at the wave length of 450 nm. The negative control value was considered as 0.05. Sample's value ≥ 2.1 times of the negative control was considered positive. Each sample was examined for more than twice. The final value was presented as mean \pm SD, after analyzing with *t* test.

Cytologic morphology

Cytologic morphological changes were observed under the Olympus optical microscope and transmission electron microscope.

Statistical analysis

Results were expressed as follows. Statistical analyses were carried out with the software package SPSS11.0. The means of the groups were compared with repeated measures analysis of variance. $P < 0.05$ was considered statistically significant.

RESULTS

Growth arrest of Bxpc-3 cell in vivo

No significant difference in absorbance value of the control group and the NS-ODN group was observed ($F = 0.013$, $P > 0.05$), whereas a significant difference was observed compared to the Ans-hTERT group ($F = 35.347$, $P < 0.01$), thus indicating an obvious inhibition of cell growth caused by Ans-hTERT (Tables 1 and 2). This

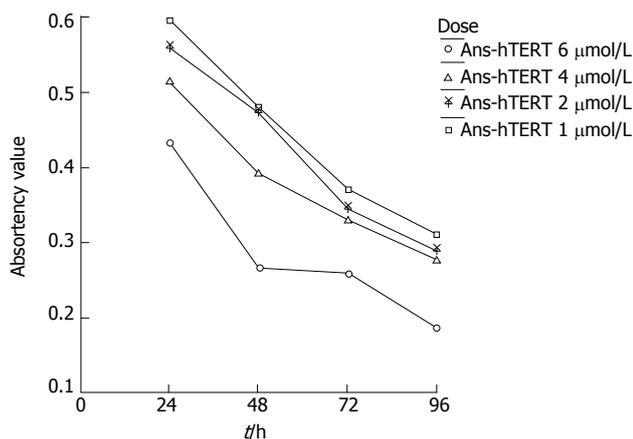


Figure 1 The absorbency value was reduction, companied by the increase of ans-hTERT's time and dose.

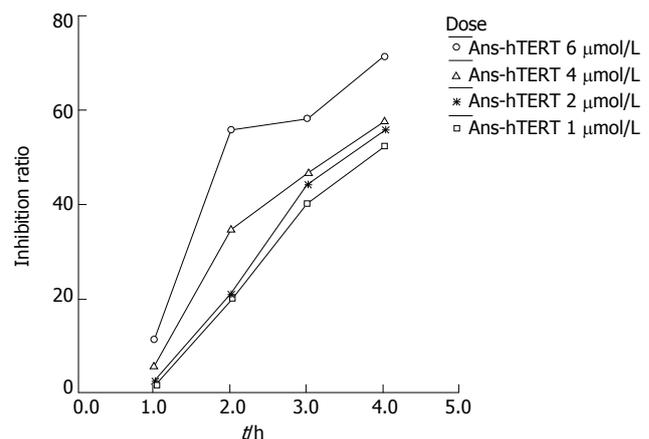


Figure 2 The inhibition ratio was enhancement, companied by the increase of ans-hTERT's dose and time.

Table 1 Absorbance values of the different groups (mean \pm SD)

Groups	24 h	48 h	72 h	96 h
Control	0.48 \pm 0.10	0.60 \pm 0.10	0.61 \pm 0.03	0.65 \pm 0.05
NS-ODN (6 μ mol/L)	0.50 \pm 0.07	0.61 \pm 0.19	0.60 \pm 0.08	0.66 \pm 0.19
ANS-ODN (1 μ mol/L)	0.60 \pm 0.03	0.48 \pm 0.06	0.37 \pm 0.03	0.31 \pm 0.05
ANS-ODN (2 μ mol/L)	0.56 \pm 0.02	0.47 \pm 0.09	0.34 \pm 0.04	0.29 \pm 0.03
ANS-ODN (4 μ mol/L)	0.51 \pm 0.10	0.39 \pm 0.08	0.33 \pm 0.04	0.28 \pm 0.03
ANS-ODN (6 μ mol/L)	0.43 \pm 0.10	0.27 \pm 0.02	0.26 \pm 0.06	0.19 \pm 0.00

Table 2 Ratio of inhibition of Ans-hTERT group (mean \pm SD)

ANS-ODN	24 h	48 h	72 h	96 h
1 μ mol/L	1.2 \pm 6.4	19.6 \pm 10.6	39.9 \pm 4.8	52.0 \pm 8.1
2 μ mol/L	2.5 \pm 7.5	21.1 \pm 15.4	43.9 \pm 6.6	55.4 \pm 4.6
4 μ mol/L	5.6 \pm 9.7	34.6 \pm 13.4	46.4 \pm 6.1	57.3 \pm 5.3
6 μ mol/L	11.4 \pm 19.2	55.6 \pm 3.6	57.9 \pm 9.0	71.3 \pm 0.4

growth inhibitory effect was dose- and time-dependent (Figures 1 and 2).

Effect of antisense hTERT ODNs on induction of Bxpc-3 cells apoptosis

After incubation of the cells with 6 μ mol/L ans-hTERT for 72 h, the G₀/G₁ stage cell had obvious difference (3.6 \pm 1.6% vs 13.6 \pm 1.0%, $t = 9.522$, $P = 0.01$), and a sub-G₁ stage cell apoptosis peak appeared in front of G₁ stage.

Effect of antisense hTERT ODNs on telomerase activity in Bxpc-3 cells

After incubation of the cells with 6 μ mol/L ans-hTERT for 72 h, a significant increased telomerase activity of ans-hTERT group was observed (1.209 \pm 0.308 vs 0.447 \pm 0.087, $t = 4.128$, $P < 0.05$) compared to the control group. The ratio of inhibition of the telomerase activity was 63% (Ratio of inhibition = 1 - ans-hTERT group/control group \times 100%).

Change in cytologic morphology

After incubation of the cells with 6 μ mol/L ans-hTERT

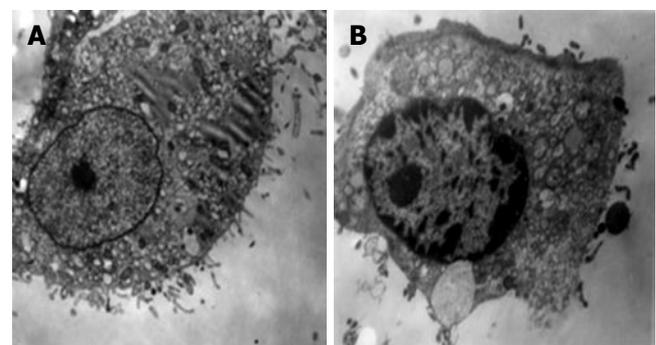


Figure 3 Chromatin of nucleolus congregated and distributed inside karyotheca, appearing half-moon type. A: Bxpc-3 cell control group; B: ans-hTERT group.

for 72 h, the chromatin in the nucleolus was congregated, distributed in the karyotheca and appeared as half-moon type. The villus on the surface of karyotheca died away and came into being the big and small varied apophysis of vesicular shape. The cell appeared undergone apoptosis (Figure 3A and B).

DISCUSSION

Telomeres form the ends of eukaryotic chromosomes consisting of an array of tandem repeats of hexanucleotide 5'-TTAGGG-3'. Telomeres protect the chromosomes from DNA degradation, end-to-end fusions, rearrangements and maintain nuclear structure^[24]. Human telomerase is a ribonucleoprotein complex, composed of a catalytic reverse transcriptase subunit (hTERT), an RNA component (hTR) that serves as a template for the synthesis of telomeric repeats, and an associated protein subunit (TP1)^[10,12,25]. It adds telomeric repeats to the 3' end of telomeric DNA. This telomere stabilization by telomerase can lead to unlimited cell proliferation. Therefore, inhibition of telomerase activity will be a new therapeutic modality for cancer^[26-32]. In most researches, the target gene was hTR, which is the replication template for telomere, but not the regulatory region of hTERT, the most important region which regulates the activity

of telomerase. Studies have reported that anti-hTR complementary to the template region of hTR is sufficient to inhibit P3 cell telomerase activity and cell proliferation *in vitro*^[3,33]. But the expression level of hTR has no relation with the tumor's development. Most current studies have proposed that reactivation of telomerase is a critical step in tumorigenesis^[34-38]. There is a close correlation between hTERT and telomerase activity. Several researchers, using ISH techniques, have demonstrated high levels of hTERT expression in malignant tissues but not in non-malignant tissues. Du *et al*^[39] showed that the telomerase activity was correlated with the cell growth. Our findings also revealed a good correlation between the inhibition of telomerase activity and reduction in cell growth. However, in our study, the cell growth inhibition was mainly the result of cell cycle arrest, not the increased cell apoptosis rate.

In conclusion, growth of pancreatic cancer cell Bxp-3 is inhibited by ans-hTERT oligodeoxynucleotides in a dose- and time-dependent manner, which is associated with the cell cycle accumulation in G₀/G₁ phase. The cell ratio of G₀/G₁ phase increased from 2.70% to 14.69%, the cell ratio of S phase decreased from 72.7% to 51.0% and a sub-G₁ stage cell apoptosis peak appeared in front of G₁ stage. Our results showed that ans-hTERT oligodeoxynucleotides can effectively inhibit hTERT gene expression, decrease telomerase activity, and trigger apoptosis. It is likely to raise the possibility that antitumor effect of ans-hTERT oligodeoxynucleotides occurs through following two pathways: (1) a short-term effect on apoptosis is induced rapidly by as-hTERT^[40]; and (2) a long-term effect on telomerase activity is inhibited, and cell death is caused when telomere length is critically shortened by telomeric DNA. Apoptosis induction may be one of the potential mechanisms of ans-hTERT oligodeoxynucleotides-mediated inhibition for tumor cell growth. Continuous ans-hTERT oligodeoxynucleotides treatment might shorten the telomere to a size that leads to cell senescence. The treatment with ans-hTERT oligodeoxynucleotides may be a potential strategy for cancer with telomerase activity.

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Depression of biofilm formation and antibiotic resistance by *sarA* disruption in *Staphylococcus epidermidis*

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Abstract

AIM: To study the effects of disruption of *sarA* gene on biofilm formation and antibiotic resistance of *Staphylococcus epidermidis* (*S. epidermidis*).

METHODS: In order to disrupt *sarA* gene, the double-crossover homologous recombination was applied in *S. epidermidis* RP62A, and tetracycline resistance gene (*tet*) was used as the selective marker which was amplified by PCR from the pBR322 and inserted into the locus between *sarA* upstream and downstream, resulting in pBT2 Δ *sarA*. By electroporation, the plasmid pBT2 Δ *sarA* was transformed into *S. epidermidis*. Gene transcription was detected by real-time reverse transcription-PCR (RT-PCR). Determination of biofilm was performed in 96-well flat-bottomed culture plates, and antibiotic resistance was analyzed with test tube culture by spectrophotometry at 570 nm respectively.

RESULTS: A *sarA* disrupted strain named *S. epidermidis* RP62A Δ *sarA* was constructed, which was completely defective in biofilm formation, while the *sarA* complement strain RP62A Δ *sarA* (pHPS9*sarA*) restored the biofilm formation phenotype. Additionally, the knockout of *sarA* resulted in decreased erythromycin and kanamycin resistance of *S. epidermidis* RP62A. Compared to the original strain, *S. epidermidis* RP62A Δ *sarA* had an increase of the sensitivity to erythromycin at 200-400 μ g/mL and kanamycin at 200-800 μ g/mL respectively.

CONCLUSION: The knockout of *sarA* can result in the defect in biofilm formation and the decreased erythromycin and kanamycin resistance in *S. epidermidis* RP62A.

INTRODUCTION

Staphylococcus epidermidis (*S. epidermidis*), a normal inhabitant of human skin and mucous membranes, is the predominant cause of foreign-body-associated infections. The pathogenesis of *S. epidermidis* infections is correlated with its ability to form biofilms on polymer surfaces^[1,2]. Biofilm formation proceeds in two phases^[3,4]. Primary attachment of bacterial cells to a polymer surface is a complex process influenced by a variety of factors, including hydrophobic interactions, presence of host proteins, and specific bacterial proteins and polysaccharides like the capsular polysaccharide adhesin, the autolysin AtlE, and other staphylococcal surface proteins^[5,6]. This is followed by the second phase leading to accumulation of bacteria in a multilayered biofilm embedded in an amorphous glycocalyx. This phase is a multifactorial process that is influenced by a number of factors. Among the most important of these is polysaccharide intercellular adhesion (PIA). Synthesis of polysaccharide intercellular adhesin is essential for bacterial cell accumulation because it mediates cell-to-cell adhesion of proliferating cells^[7,8]. PIA consists of two polysaccharide species which are composed of β -1, 6-linked 2-deoxy-2-amino-D-glucopyranosyl residues containing non-N-acetylated amino groups, phosphate, and succinate. The enzymes required for polysaccharide intercellular adhesin synthesis are encoded within the *icaADBC* operon, mutation of which results in a reduced capacity to form a biofilm in *S. epidermidis*^[7]. *SarA*, a central regulatory element that controls the production of *Staphylococcus aureus* (*S. aureus*) virulence factors, is essential for the synthesis of PIA and the ensuing biofilm development in this species^[9,10]. Based on the presence of a *sarA* homolog, we hypothesized that SarA could also be involved in the regulation of the biofilm formation process in *S. epidermidis*. To elucidate the possible role of SarA in biofilm formation, we used

Table 1 Strains and plasmids used in the study

Strains or plasmids	Relevant characteristics	Sources or references
Strains		
<i>S. epidermidis</i> ATCC12228	Biofilm negative	ATCC
<i>S. epidermidis</i> RP62A (ATCC 35984)	Biofilm positive Kan ^r , Ery ^r , Ap ^r	ATCC
<i>S. epidermidis</i> RP62AΔ <i>sarA</i>	<i>sarA</i> deletion on the chromosome, Tet ^r , <i>sarA::tet</i>	This study
<i>S. epidermidis</i> RP62A (pHPS9 <i>sarA</i>)	<i>S. epidermidis</i> RP62AΔ <i>sarA</i> harboring pHPS9 <i>sarA</i>	This study
<i>S. aureus</i> RN4220	Restriction-negative, intermediate host for plasmid transfer from <i>E. coli</i> to <i>S. epidermidis</i>	[14]
<i>E. coli</i> DH5α	φ80 <i>dlacZ</i> Δ <i>M15 recA1 endA1 gyrA96 thi-1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>supE44 relA1 deoR</i> Δ(<i>lacZYA-argF</i>) <i>U169</i>	Promega Co.
Plasmids		
pBR322	Donator of <i>tet</i> gene (Tet ^r). Ap ^r Tet ^r	[15]
pBluescriptSK	<i>E. coli</i> cloning vector. Ap ^r	[11]
pBT2	Temperature-sensitive shuttle vector. Ap ^r (<i>E. coli</i>) Cm ^r (<i>Staphylococcus</i>)	[16]
pBTΔ <i>sarA</i>	Integration vector for homologous recombination of the Δ <i>sarA</i> gene in <i>S. epidermidis</i> ; <i>tet</i> inserted into <i>sarA</i> locus as resistance selection marker	This study
pHPS9	Expression shuttle vector	[17]
pHPS9 <i>sarA</i>	pHPS9 inserted <i>sarA</i> gene	This study

tet^r, resistance to tetracycline; kan^r, resistance to kanamycin; ery^r, resistance to erythromycin; Ap^r, resistance to ampicillin; Cm^r, resistance to chloramphenicol.

a genetic approach and constructed an *S. epidermidis sarA::tet* knockout mutant of the biofilm-forming strain *S. epidermidis* RP62A. Biofilm formation and *ica* expression of the mutant were compared with the phenotypes of the corresponding wild-type strain and a complemented strain that carried a *sarA* copy in an expression vector.

We were interested in the potential role of SarA in the response of *S. epidermidis* to antimicrobial agents. Therefore, we used this *sarA* knockout mutant and determined its influence on erythromycin and kanamycin resistances in *S. epidermidis* RP62A (i.e. ATCC35984).

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth medium

The bacterial strains and plasmids used in this study are listed in Table 1. Tryptic soy broth (TSB) was used to grow *Staphylococcus* strains. Luria-Bertani (LB) was used for *E. coli*. Chloramphenicol was used at 15 μg/mL, ampicillin at 100 μg/mL and tetracycline at 10 μg/mL. Bacteria were grown on Congo red agar (CRA) plates, which were composed of TSB agar supplemented with 5% sucrose (A.R.) and 0.8 mg of Congo red/L (A.R.). All DNA manipulations and handling of *E. coli* were performed in accordance with standard protocols^[11]. Manipulations with *Staphylococcus* were performed as described previously^[12,13].

Construction of plasmids and *sarA::tet* allele replacement

In order to analyze its function in *S. epidermidis*, *sarA* was replaced by a tetracycline resistance gene (*tet*) by homologous recombination. The upstream of 836 bp, fragment 1, was amplified using primer pairs (1) 5'-ACGAAGCTTC TGTAACATCT AGTGACAA-3' and (2) 5'-ACGCTGCAGT TTAATCTGTC AGCATAAGTG-3' with *HindIII* and *PstI* respectively. The downstream of 857 bp, fragment 2, was amplified using primer pairs (3) 5'-ACGCTGCAGA TTATAAACAA CCTCAAGTTG-3' and (4) 5'-ACGGAATTCG GGCATCATTG CGAGTGA-3' with *PstI* and *EcoRI* respectively. The two fragments were cloned into the multi-

cloning region of temperature-sensitive *E. coli-Staphylococcus* shuttle vector pBT2^[16], resulting in plasmid pBT2-1. A fragment of 1276 bp containing the entire *tet* gene was amplified by PCR from the pBR322, using the primers (5) 5'-CGCGCGGCCG CTTCTCATGT TTGACAGCTT-3' and (6) 5'-GCGAGATCTT CAGGTTCGAGG TGGCC-3'. The *tet* gene was inserted into the vector pBT2-1, resulting in plasmid, pBT2Δ*sarA*. Following passage through the restriction-negative strain *S. aureus* RN4220, pBT2Δ*sarA* was reisolated and transformed into *S. epidermidis* RP62A by electroporation. Replacement of the chromosomal *S. epidermidis* RP62A *sarA* wild-type gene was obtained by double-crossover integration of the *sarA::tet* insert of pBT2 Δ*sarA* following a temperature shift to the nonpermissive temperature (42°C) of the shuttle vector^[18]. Tetracycline-resistant and chloramphenicol-sensitive colonies were isolated. The *sarA::tet* integrations were confirmed by PCR detection (Figure 1 step 4) and the nucleotide sequencing was carried out by Shanghai Bioasia.

RNA purification and RT-PCR

RNA purification, real-time reverse transcription-PCR (RT-PCR) and analysis of RT-PCR data were performed as previously described^[19], with the following oligonucleotide primer pairs: for *gyrB* transcript, (7) 5'-TTATGGTGCT GGACAGATAC A-3' and (8) 5'-CACCGTGAAG ACCGCCAGAT A-3'; for *icaA* transcript, (9) 5'-AACAAGTTGA AGGCATCTCC-3' and (10) 5'-GATGCTTGTT TGATTCCCT-3'. The *gyrB* gene was compositively expressed in *S. epidermidis* and thus used as an internal standard in these RT-PCR experiments^[19].

Phenotypic assay on biofilm production

The biofilm production assay was performed by cultivation of the *S. aureus* and *S. epidermidis* strains on CRA plates as described by Freeman *et al*^[14]. The black, rough and dry colonies on CRA plates indicated the biofilm production. In contrast, the biofilm-negative strains formed red, smooth colonies.

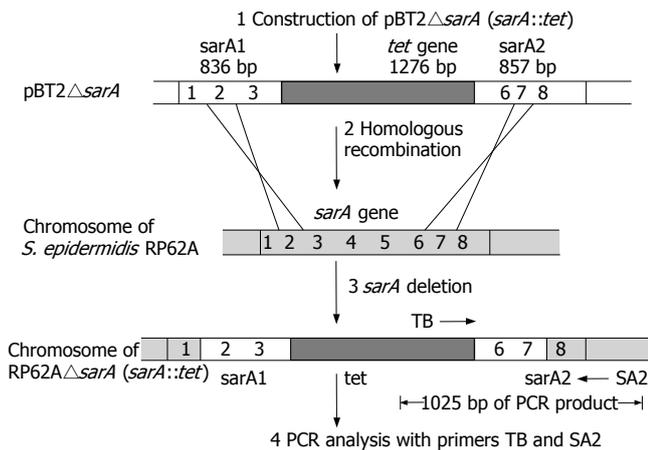


Figure 1 Disruption and detection of chromosomal *sarA* locus in *S. epidermidis*. 1. Insertion of *tet* gene into *sarA* locus to form recombinant plasmid pBT Δ *sarA*. 2. Double-exchange homologous recombination between chromosomal DNA and plasmid DNA. 3. Locus *sarA* of chromosome was destroyed by DNA recombination. 4. Detection of recombination by PCR amplification with primers TB and SA2.

Quantitative determination of biofilm formation

Strains were cultivated overnight (16 h) in 96-well flat-bottomed tissue culture plates at 37°C in TSB growth medium. Based on the optical densities (OD₅₇₀) of the biofilm, the strains were classified as non-adherent strains (OD₅₇₀ ≤ 0.120).

Determination of antibiotic susceptibilities

Three antibiotics were used to determine the impact of *sarA* mutant on *S. epidermidis* resistance to erythromycin or kanamycin. Erythromycin or kanamycin was added into test tubes with a twofold serial dilution scheme (0, 25, 50, 100, 200, 400, 800 μg/mL)^[20,21]. The inoculum was derived from overnight liquid cultures in TSB and was inoculated into shaking liquid cultures containing erythromycin or kanamycin. After overnight incubation (16 h) at 37°C, these inocula were determined with spectrophotometer at 570 nm.

RESULTS

Disruption of *sarA* gene on the chromosome DNA of *S. epidermidis* RP62A

A strain termed *S. epidermidis* RP62A Δ *sarA* (*sarA*::*tet*) derived from RP62A with an allele replacement of the *sarA* gene was obtained (Figure 1).

PCR analysis of DNA from strains RP62A and RP62A Δ *sarA* was performed with two primers as shown below: TB: 5'-ACGGAGCTCA AGCCTATGCC TACAGCA-3' (in the central part of the *tet* gene) (Figure 1); SA2: 5'-ACGGAATTCG GGCATCATTG CGAGTGA-3' (next to the downstream of *sarA2* fragment) (Figure 1). The fragment was obtained as shown in Figure 2, which indicated that allelic replacement had taken place. The PCR-amplified fragment was further demonstrated by DNA sequencing, and the result revealed that a 1025 bp fragment was composed of part of *tet* gene and chromosomal DNA (the datum of DNA sequence not shown).

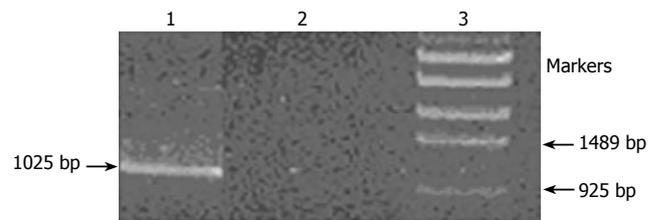


Figure 2 Detection of *sarA* gene locus in chromosome DNA by PCR with primers SA2 and TB. Lane 1, The product was amplified from *S. epidermidis* RP62A Δ *sarA* with *tet* insertion; lane 2, No product was amplified from *S. epidermidis* RP62A without *tet* insertion; lane 3, DNA markers.

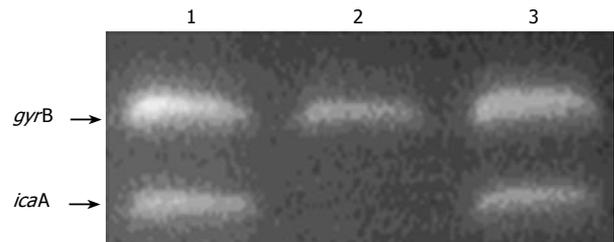


Figure 3 Identification of the genes *icaA* and *gyrB* transcription in *S. epidermidis* by RT-PCR analysis. 1. *S. epidermidis* RP62A; 2. *S. epidermidis* RP62A Δ *sarA*; 3. *S. epidermidis* RP62A Δ *sarA* (pHPS9*sarA*).

Repression of *icaA* transcription in *S. epidermidis* RP62A by disruption of *sarA*

To investigate whether the transcription of *ica* operon expression was altered in the *sarA* mutant strain, RT-PCR was used to measure *icaA* transcription in variants grown in TSB. Total RNA of the original RP62A strain, its *sarA* mutant and corresponding complementary strain were isolated at early exponential and mid-log exponential phases, as the expression of *ica* operon was at maximum during this period. After treatment with DNase to remove contaminant DNA, RNA was reverse transcribed in the presence and absence of reverse transcriptase. The level of expression of *icaA* was normalized to *gyrB* expression^[19]. Our results showed that the level of *ica* operon transcription was apparently reduced in the *sarA* mutation compared to that of the wild-type strain at exponential and mid-log exponential phases (Figure 3, Lane 2). Interestingly, in the *sarA* complementation strain, designated as RP62A Δ *sarA* (pHPS9*sarA*), the *icaA* transcription was activated (Figure 3, lane 3). Consistent with this, at the phenotypic level, the capacity of RP62A Δ *sarA* (pHPS9*sarA*) to form biofilm was restored in TSB. These data suggested that the gene of *sarA* in the strain of RP62A is responsible for activating *ica* operon expression.

Depression of biofilm formation of *S. epidermidis* RP62A by inactivation of *sarA*

Phenotypic assay on biofilm production: The *S. epidermidis* RP62A strain formed typical black, rough colonies after 24 h of incubation. The non-slime producing *S. epidermidis* ATCC 12228 formed smooth, red colonies. RP62A Δ *sarA* strain produced smooth, red colonies after 24 h, demonstrating the mutant was biofilm-negative. As to the

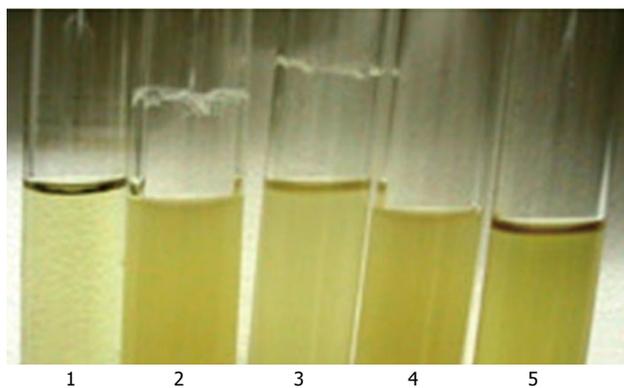


Figure 4 Biofilm formation on the glass surface in the tube culturing *S. epidermidis*. 1. TSB medium not inoculated; 2. Biofilm-positive strain RP62A (biofilm-forming); 3. *sarA* complemented strain RP62A Δ *sarA* (pHPS9*sarA*) (biofilm-forming); 4. *sarA* deletion strain RP62A Δ *sarA* (non-biofilm-forming); 5. Biofilm-negative strain ATCC12228 (non-biofilm-forming).

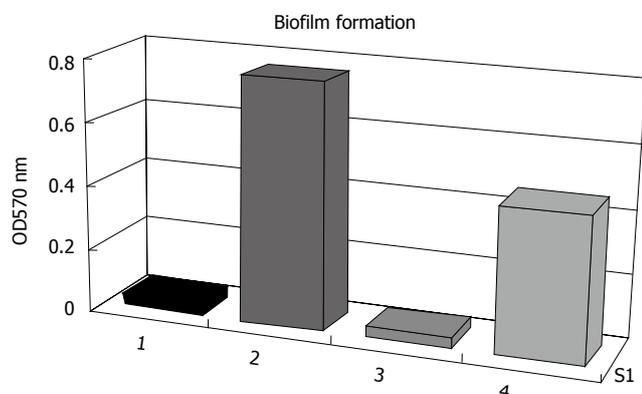


Figure 5 Biofilm formation on polystyrene tissue culture plates of *S. epidermidis* in TSB medium. 1. biofilm-negative strain *S. epidermidis* ATCC12228; 2. original strain *S. epidermidis* RP62A; 3. *sarA::tet* insertion mutant *S. epidermidis* RP62A Δ *sarA*; 4. complemented strain *S. epidermidis* RP62A Δ *sarA* (pHPS9*sarA*).

RP62A Δ *sarA* (pHPS9*sarA*) strain, black, rough colonies phenotype was restored.

Biofilm formation on a glass surface of the shaking tube with overnight culture: The *sarA* mutant showed loss of the ability to produce a ring of biofilm adherent to the glass wall at the air-liquid interface (Figure 4, Lane 2). While the complementary strain RP62A Δ *sarA* (pHPS9*sarA*) displayed a biofilm positive phenotype similar to that of the wild-type strain (Figure 4, Lane 3).

Quantitative determination of biofilm formation: All strains were then tested of their ability to form biofilms on polystyrene surfaces. The isolates were grown overnight in 96-well flat-bottomed tissue culture using TSB as growth medium. As shown in Figure 5, the biofilm formation of *S. epidermidis* RP62A was biofilm-positive when the strain was grown in TSB. In contrast, the *S. epidermidis* RP62A Δ *sarA* insertion mutant and *S. epidermidis* ATCC12228 failed to produce any detectable biofilm. In the case of *S. epidermidis* RP62A Δ *sarA* (pHPS9*sarA*), in which the deleted chromosomal locus of *sarA* was complemented by a plasmid carrying *sarA*, biofilm formation was restored.

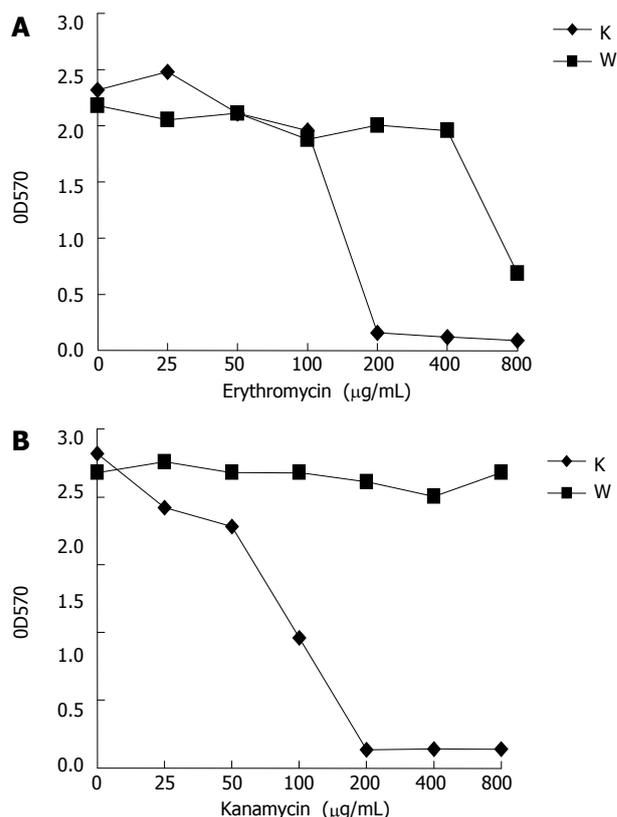


Figure 6 Response of RP62A (original) and RP62A Δ *sarA* (mutant) to two antibiotics in shaking tube cultures. The cultures of the wild type RP62A (W) and the mutant RP62A Δ *sarA* (K) were incubated in a shaking incubator (250 r/min) at 37°C for 24 h, and diluted in fresh TSB medium. Determination of optical density (OD) values was carried out at 570 nm.

Increase in some antibiotic sensitivity of *S. epidermidis* RP62A by deletion of *sarA*

The *sarA* mutation had a dramatic effect on the antibiotics resistance of strain RP62A. Two antibiotics, erythromycin and kanamycin, were investigated. The *sarA::tet* mutant demonstrated a significant increase of susceptibility along with the increasing concentration of erythromycin and kanamycin compared to its parental strain (Figure 6). At erythromycin concentrations above 200 μg/mL, RP62A Δ *sarA* did not grow, while RP62A survived at concentrations over 800 μg/mL (Figure 6A). RP62A appeared at all kanamycin concentrations investigated, whereas RP62A Δ *sarA* did not survive at concentrations over 200 μg/mL (Figure 6B).

DISCUSSION

To investigate the impact of *sarA* on biofilm formation, we constructed a *sarA* mutant of *S. epidermidis* RP62A. At the phenotypic level, the *sarA* mutant revealed a biofilm-negative phenotype, and the capacity of biofilm formation was restored when *sarA* mutant strains were complemented by a plasmid carrying *sarA*. RT-PCR was used to measure the transcription of *icaA* in variants grown in TSB. Consistent with the biofilm-negative phenotype, the results showed that the *sarA* mutation caused a significant

repression of the *ica* operon transcription compared with that of the wild-type strains. The absence of an identifiable *sigB*-consensus binding site on the upstream of the *ica* operon in *S. aureus* has suggested that *sigB* may not regulate *ica* operon directly^[22], and the foundation of the SarA-consensus binding site implied that SarA controls directly the transcription of *ica* operon and subsequently regulates biofilm formation^[23,24]. However, it is possible that both SigB and SarA are also involved in the posttranscriptional regulation of PIA synthesis in *S. epidermidis* according to previous reports.

The knockout of *sarA* resulted in decreased erythromycin and kanamycin resistance in *S. epidermidis* RP62A. This implies that a protein(s) whose production is controlled by SarA is involved in resistance to these antibiotics. However, the exact role that SarA plays in the response of *S. epidermidis* to multiple antibiotics deserves further attention.

In conclusion, the current studies demonstrate that SarA is typically associated with the transcription of the *ica* operon, the capacity of biofilm-formation and antibiotic resistance of *S. epidermidis*. To answer the question of whether SarA is directly or indirectly involved in those processes, more experimental work, including primer extension analyses under different growth conditions, is needed.

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BASIC RESEARCH

Differentiation of human umbilical cord blood stem cells into hepatocytes *in vivo* and *in vitro*

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Abstract

AIM: To study the condition and potentiality of human umbilical cord blood stem cells (HUCBSC) to differentiate into hepatocytes *in vivo* or *in vitro*.

METHODS: In a cell culture study of human umbilical cord blood stem cell (HUCBSC) differentiation, human umbilical cord blood mononuclear cells (HUCBMNC) were separated by density gradient centrifugation. Fibroblast growth factor (FGF) and hepatocyte growth factor (HGF) and the supernatant of fetal liver were added in the inducing groups. Only FGF was added in the control group. The expansion and differentiation of HUCBMNC in each group were observed. Human alpha fetoprotein (AFP) and albumin (ALB) were detected by immunohistochemistry. In the animal experiments, the survival SD rats with acute hepatic injury after carbon tetrachloride (CCL₄) injection 48 h were randomly divided into three groups. The rats in group A were treated with human umbilical cord blood serum. The rats in group B were treated with HUCBMNC transplantation. The rats in group C were treated with HUCBMNC transplantation followed by intraperitoneal cyclophosphamide for 7 d. The rats were killed at different time points after the treatment and the liver tissue was histopathologically studied and human AFP and ALB detected by immunohistochemistry. The human X inactive-specific transcript gene fragment in the liver tissue was amplified by PCR to find human DNA.

RESULTS: The results of cell culture showed that adherent cells were stained negative for AFP or ALB in control group. However, the adherent cells in the inducing groups stained positive for AFP or ALB. The result of animal experiment showed that no human AFP or ALB positive cells present in the liver tissue of group

A (control group). However, many human AFP or ALB positive cells were scattered around sinus hepaticus and the central veins of hepatic lobules and in the portal area in group B and group C after one month. The fragment of human X chromogene could be detected in the liver tissue of groups B and C, but not in group A.

CONCLUSION: Under certain conditions HUCBSC can differentiate into liver cells *in vivo* and *in vitro*.

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Key words: Umbilical cord blood; Stem cell; Liver failure; Cell transplantation; Cell differentiation

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<http://www.wjgnet.com/1007-9327/12/4014.asp>

INTRODUCTION

Umbilical cord blood (UCB) remains in the placenta and umbilical cord after birth. The placenta and umbilical cord blood is usually discarded after delivery as a medical waste. The presence of hematopoietic stem cells (HSC) in UCB was first demonstrated in 1974. There is evidence that UCB is a rich source of HSC. However, it was not until 1989 that experimental and clinical studies were published, indicating that UCB can be used in clinical settings. Since then, UCB has been used as an alternate source of HSC for transplantation. Some data suggest that UCB is a better source of HSC than bone marrow (BM)^[1,2]. It has been found that multipotent adult progenitor cells can differentiate into hepatocyte-like cells *in vitro*^[3]. Neurons, astrocytes and oligodendrocytes can be propagated *in vitro* from UCB cells^[4,5], indicating that stem cells can differentiate into hepatocytes^[6,7]. These experiments studied the conditions and potentiality of human umbilical cord blood cells (HUCBSC) to differentiate into hepatocyte *in vivo* or *in vitro*.

MATERIALS AND METHODS

Reagents

Fetal bovine serum (Hyclone, USA), Dulbecco's modi-

fied Eagle's medium (DMEM) (Gibco, USA), β -fibroblast growth factor (FGF) and hepatocyte growth factor (HGF) (Preprotech, USA), immunohistochemistry kit (PV900, Beijing), alpha fetoprotein (AFP) and mouse anti human monoclonal antibody (Maixin, Foochow), albumin and (ALB) rabbit anti human polyclonal antibody (DAKO), protease K, NaI, glass powder, Taq DNA polymerase, $10 \times$ buffer, random primer (2.5 mmol/L , dNTP) were provided by Promega Co.

Collection and storage of umbilical cord blood

Immediately after birth of the baby, the umbilical cord was cut off and the baby was separated from the placenta and mother. A sterile needle was inserted into the umbilical vein and UCB was drawn into a sterile blood collection bag containing ACD-B or heparin anticoagulant. Once the collection was completed, the specimen was packaged and sent to blood bank for processing and storage at low temperatures. The maternal blood sample was also collected for infectious disease analysis.

Separation of human umbilical cord blood mononuclear cells

Human umbilical cord blood mononuclear cells (HUCBMNC) were separated from UCB by density gradient centrifugation. When cell motility rate was more than 95%, the cell concentration was adjusted to 5×10^5 /mL.

Cell Culture

FGF 2.5 ng/mL and HGF 20 ng/mL and supernatant of fetal liver (5%, V/V) were added into the HUCBMNC culture flasks in the inducing groups. Only FGF was added into the HUCBMNC culture flasks in the control group. The expansion and differentiation of HUCBMNC in each group were observed. Human AFP and ALB were detected by immunohistochemistry.

Animal experiment

Adult SD rats, weighing $180 \pm 20 \text{ g}$ were provided by the Animal Laboratory of the Second Xiangya Hospital. Carbon tetrachloride (CCL_4 , $0.7 \text{ mL}/100 \text{ g}$, 10%) was intraperitoneally injected into SD rats to establish an acute hepatic injury model. Surviving rats 48 h after CCL_4 injection were randomly divided into three groups. Rats in group A were treated with 1 mL human umbilical cord blood serum. Rats in group B were treated with 1 mL HUCBMNC. Rats in group C were treated with 1 mL HUCBMNC followed by intraperitoneal cyclophosphamide $2 \text{ mg}/100 \text{ g}$ per d for 7 d. The general state of the rats in each group was observed.

Detection of human AFP and ALB in rat liver tissue

The rats were killed at different time points after treatment and the liver tissue was histopathologically studied and detected for human AFP and ALB by immunohistochemistry.

PCR amplification of human X inactive specific transcript gene

The primers of PCR were designed according to human X

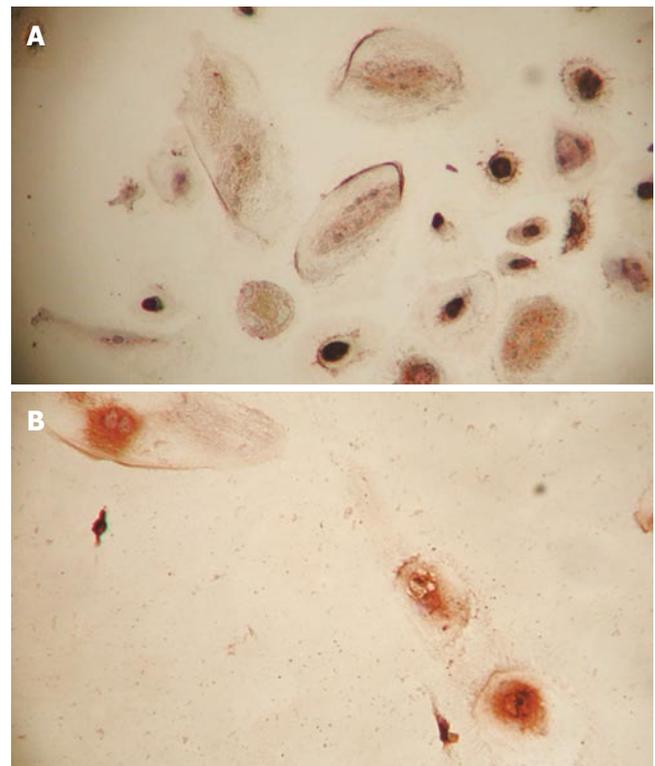


Figure 1 Cells positively stained for AFP (A) and ALB (B) in inducing groups (400 \times).

inactive specific transcript (XIST) gene (upstream primer P1: 5'- TTA CTG GCTGTATTGCCTTGC- 3'; downstream primer P2: 5'- ATTATCTCCA CCGCTTCACT -3'). DNA was extracted from the rat liver tissue imbedded in paraffin and amplified by PCR. Gel electrophoresis was performed to find whether human DNA was present in the rat liver tissue.

RESULTS

Expansion and differentiation of HUCBSC

The result showed that most adherent cells had fusiform shape. No polygon shape cells were found in the control group. However, round shape, spherical and polygon shape adherent cells as well as fusiform cells were found in the inducing groups.

Immunohistochemistry staining of AFP and ALB

The adherent cells in the control group were stained negative for AFP or ALB by immunohistochemistry, thus these adherent cells were most probably fibroblasts. The adherent cells in the inducing groups were stained positive for AFP or ALB by immunohistochemistry (Figures 1A and 1B) and therefore they might be hepatocytes.

Influence of two different decoagulants on HUCBMNC

Heparin or natrium citricum was used as a decoagulant when UCB was collected. The expansion and differentiation of HUCBMNC anti-coagulated with heparin were better than those of HUCBMNC anti-coagulated with natrium citricum.

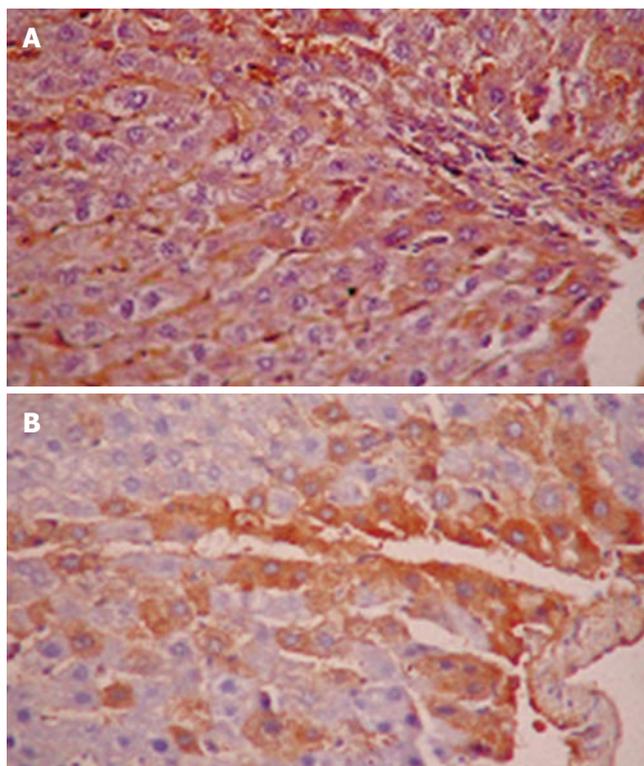


Figure 2 Expression of human ALB (A) and AFP (B) in rat liver tissue (200 ×).

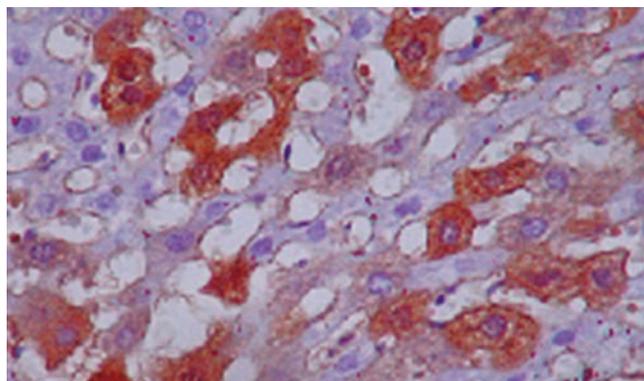


Figure 3 Human ALB positively conjugated nuclear cells in rat liver tissue (400 ×).

Effects of two different inducing factors

Two different inducing factors, HGF and fetal liver supernatant were used to induce differentiation of HUCBSC into hepatocytes. The inducing effect of each was compared. The result showed that the inducing effect of HGF was better than that of fetal liver supernatant.

Expression of human AFP and ALB in rat liver tissue

No human AFP or ALB positive cells were found in rat liver tissue of group A (control group) by immunohistochemistry. However, many human AFP or ALB positive cells were scattered around sinus hepaticus and central veins of hepatic lobules and portal area in groups B and C after one month (Figures 2A and 2B). Moreover, a few human ALB positively conjugated nuclear cells were found in rat liver tissue of group C (Figure 3).

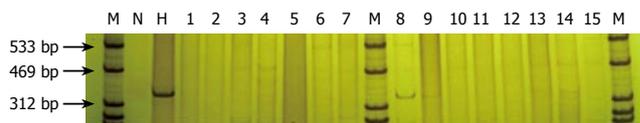


Figure 4 PCR-amplified DNA. M: 100 bp marker; N: Negative control, the products of PCR templated by water; H: Positive control, the products of PCR templated by human fetal liver tissue DNA; 1-5: DNA extracted from rat liver tissue embedded in paraffin in group A; 6-10: DNA extracted from rat liver tissue embedded in paraffin in group B; 11-15: DNA extracted from rat liver tissue embedded in paraffin in group C.

Fragment of human X chromogene in rat liver

XIST fragment of human X chromogene could be detected in rat liver tissue of groups B and C by DNA extraction and PCR amplification, but no human X chromogene fragment was found in group A (Figure 4).

DISCUSSION

Stem cells present in adult BM and UCB are undifferentiated long-lived cells that have the ability to proliferate extensively and maintain the ability to differentiate into multiple cell types including bone cells, cartilage cells, fat cells, tendon cells, muscle cells, marrow stromal cells, astrocytes, *etc.* Potential liver-cell progenitors have been identified from BM, peripheral blood, UCB, fetal liver, adult liver and embryonic stem cells. Differences and similarities are found among cells isolated from rodents and humans^[8].

Stem cells are present in UCB and possess several typical traits^[9]. Clonal culture of fluorescence-activated cell sorter CD34+ UCB and BM cells revealed a higher incidence of colony-forming cells with greater proliferation capacity in UCB than BM CD34+ cells. UCB CD34+ cells also demonstrated a higher secondary plating efficiency than BM cells. Rats transplanted with UCB mononuclear cells showed significantly higher levels of chimerism than those transplanted with BM mononuclear cells.

Recipients of UCB transplants from HLA-identical siblings have a lower incidence of acute and chronic graft-versus-host disease than recipients of BM transplants from HLA-identical siblings^[10]. Immature human UCB cells with high proliferative, replating, *ex vivo* expansion and mouse NOD/SCID engrafting ability can be stored frozen for > 15 years, and efficiently retrieved, and most likely remain effective for clinical transplantation^[11]. Intravenous injection of adult BM cells in FAH-/-mice, an animal model of tyrosinemia type I, could rescue the mice and restore their liver biochemical function. Within BM, only rigorously purified HSCs give rise to donor-derived hematopoietic and hepatic regeneration^[12].

Adult human liver cells can be derived from stem cells originating in the BM or circulating outside the liver, so that blood-system stem cells could be used clinically to generate hepatocytes for replacing damaged tissue^[13]. BM-derived hepatocyte stem cells integrate with hepatic cell plates and differentiate into mature hepatocytes. In a culture system simulating liver regeneration and containing cholestatic serum, these cells differentiate into mature hepatocytes and metabolize ammonia into urea depending

on a yet non-defined humoral signal existing in cholestatic serum. Transmission electron microscopy and three-dimensional digital reconstruction have confirmed hepatocyte ultrastructure of cultured BM-derived hepatocyte stem cells^[14]. In a primary culture system supplemented with growth/differentiation factors, about 50% of UCB cells in 21-d cultures express ALB, and ALB+ cells co-express hepatocyte lineage markers. ALB-expressing cells are able to proliferate in the culture system. In the cell-transplantation model of liver-injured SCID mice, inoculated UCB cells develop into functional hepatocytes in the liver, which release human ALB into the sera of the recipient mice. Thus, human UCB is a source of transplantable hepatic progenitor cells^[15].

It was reported that human UCB cells can migrate into NOD-SCID liver and become mature hepatocytes. No cell fusion can be detected in any of the human cells found in mouse liver^[16]. Human albumin expression can be detected only in CCl₄-treated mice receiving transplants of human stem cells, and recovery is increased by administration of human HGF 48 h after CCl₄-mediated liver injury^[17]. As the cells are morphologically transformed into hepatocyte-like cells, UCB-derived human MSC can express Thy-1, c-Kit and Flt-3 on the cell surface, as well as albumin, AFP and cytokeratin-18 and 19 in the interior. About a half of the cells are found to acquire the ability to transport DiI-Ac-LDL^[18].

In this study, UCB transplantation demonstrated a good therapeutic effect on severe viral hepatitis without obvious side effects. UCB attenuated the liver lesions and reproduced hepatocytes. The effect of UCB transplantation combined with plasma exchange (PE) is much better than that of PE alone, suggesting that UCB transplantation can enhance the therapeutic effect of plasma exchange on severe viral hepatitis^[19]. After 4 wk of induction of HGF and oncostatin M, stem cells isolated from human BM and UCB showed cuboidal morphology of hepatocytes and cells expressed marker genes specific for liver cells in a time-dependent manner. Differentiated cells have *in vitro* functions of liver cells, including albumin production, glycogen storage, urea secretion, uptake of low-density lipoprotein, and phenobarbital-inducible cytochrome P450 activity^[20]. Human UCB stem cells are able to transdifferentiate into hepatocytes, to improve liver regeneration after damage and mortality rate. Intraperitoneal administration of UCB stem cells contributes to a rapid liver engraftment^[21].

CD34+ UCB cells are rich fractions in hepatic progenitor cells, and trans-differentiation from UCB cells into hepatocytes and cell fusion simultaneously occur^[22]. UCB stem cell transplantation displays good therapeutic effects on severe viral hepatitis and improves heart injury of the patients. The rat liver immunohistochemistry in this study indicated that UCB stem cells could decrease liver damage and increase hepatocellular regeneration. Human UCB stem cells can differentiate into liver cells in acutely damaged SD rat liver^[23]. Xeno-transplantation of human UCB CD34+ (hCBCD34+) cells during pre-immune stages of development in immunocompetent mice might also lead to human-mouse liver chimerism. Freshly isolated hCBCD34 (+) cells were xeno-transplanted into non-immunosuppressed

mice by both intra-blastocyst and intra-fetal injections in our study. One and four weeks after birth, immunostaining was carried out for different human-specific hepatocyte markers, such as human hepatocyte-specific antigen, human serum albumin, and human alpha-1-antitrypsin indicated the presence of human hepatocyte-like cells in the livers of transplanted animals. The results indicate that detection of human albumin mRNA further corroborates the development of pre-immune human-mouse chimeras^[24].

A new intrinsically pluripotent CD45- population called unrestricted somatic stem cells (USSC) has been isolated from human UCB. These cells grow adherently and can be expanded to at least 10¹⁵ cells while maintaining a normal karyotype and a pluripotency including hematopoietic, neural, and hepatic cell differentiation in the noninjured fetal sheep model. More than 20% albumin-producing human parenchymal hepatic cells have been obtained in the absence of cell fusion in this model. One major biological difference between USSCs and human MSC is the ease of generation of USSCs in cytokine-free cultures and the potential to generate hematopoietic cells *in vitro*. Besides their differentiation potential, USSCs can also be distinguished from MSC by their phenotype. But similar to adult and fetal MSCs, they are also nonimmunogenic and even immunosuppressive^[25].

Some studies have shown that UCB may contain some hepatic progenitors. MSC has been isolated from UCB harboring a broader potential than expected. Single clonally expanded cells isolated from either UCB or BM, can differentiate not only into osteoblasts, adipocytes, and chondrocyte-like cells (as expected for MSCs), but also into different cell types including functional hepatocytes *in vitro*. When UCB-derived cells are cultured under hepatogenic conditions all cells acquire a cuboidal morphology as opposed to the fibroblast-like morphology of undifferentiated cells. In this study, low level expression of AFP, an early developmental marker gene of hepatoblasts, was detectable by d 7 and remained detectable up to d 35. Expression of cytokeratin-18 and albumin was detectable at all time points, and the expression of tyrosine aminotransferase, a late marker gene of hepatocytes, was detectable by d 14 and increased with time of differentiation. Furthermore, by d 7, differentiated cells were stained positive for albumin. Undifferentiated cells did not express AFP or tyrosine aminotransferase, but expressed low levels of albumin and cytokeratin-18. However, undifferentiated cells were negative for albumin. After 6 wk of differentiation the hepatocyte-like cells demonstrated the ability to take up low-density lipoprotein, a function characteristic of hepatocytes, whereas undifferentiated cells failed to take up low-density lipoprotein^[26].

By seeding UCB β 2 m-c-Met+ cells (UCBCCs) on the cirrhotic fat-storing cells (CFSC)/HGF feeder layers without any cytokine added, distinct morphological changes in some UCBCCs emerged after co-cultured for 4 d, and the changes increased even more as time went on. Morphological characteristics of hepatocytes occurred after 7 d of coculture in about 38%-46% of UCBCCs, which were identified to possess hepatocyte-like functions. UCBCCs could also differentiate into hepatocyte-like cells by co-

culturing with CFSC/HGFs separately using trans-wells, although the differentiation efficiency was lower than the former. Moreover, the tests showed limited contribution of control CFSC/neo to the induction, which proved the important sustainment of nonparenchymal cells and extracellular matrices on the stem cell differentiation and prompted us to consider the insufficient factor secretion in nontransfected hepatic stellate cells only^[27].

Transplantation of human HSC in sheep has led to the establishment of human hematopoiesis and formation of a significant number of long-lasting, functional human liver cells, with some animals exhibiting levels as high as 20% of donor (human) hepatocytes 11 mo after transplantation. Human hepatocytes generated in sheep retain functional properties of normal hepatocytes, constitute hepatic functional units with the presence of human endothelial and biliary duct cells, and secrete human albumin that is detectable in circulation. Transplanting populations of HSC can efficiently generate a significant number of functional hepatic cells in sheep^[28].

Characterization of UCB-derived USSCs with the capacity to differentiate into hematopoietic and nonhematopoietic cells in the absence of cell fusion has highlighted the great potential of stem cell plasticity. A great variety of stem cell types have been defined and even the most pure marrow stem cells are highly heterogeneous. Data suggest that stem cells may exist in a continuum with continually and reversibly changing phenotype^[29].

Our study showed that with the induction of HGF or fetal liver supernatant, HUCBSC could expand *in vitro* and differentiate into polygon cells expressing AFP and ALB. These cells are most likely hepatocytes. Moreover, the expansion and differentiation of HUCBSC anti-coagulated with heparin were better than those of HUCBSC anti-coagulated with natrium citricum, suggesting that natrium citricum may be toxic for HUCBSC or heparin can promote the expansion of HUCBSC *in vitro*. Therefore, heparin is better than natrium citricum as a UCB collection decoagulant. We also showed that the role of HGF in inducing HUCBSC differentiation into hepatocytes was better than that of fetal liver supernatant, indicating that HGF can promote the expansion of HUCBSC and induce the differentiation of HUCBSC into liver cells. Thus, it is better to use HGF as an inducing factor for the differentiation of HUCBSC.

Our animal experiment showed that after one month treatment with HUCBSC, the rats with acute liver failure were positive for human AFP and ALB in liver tissue. The DNA fragment of human X chromagene could be found in rat liver tissue of HUCBSC-treated group. However, human DNA fragment could not be detected in the control group, indicating that the transfused human ALB and AFP were destroyed in the rats. Therefore, human AFP and ALB detected in rat liver tissue of HUCBSC treated-group were products of liver cells differentiated from HUCBSC. The number of positive human AFP and ALB cells found in HUCBSC-treated groups with or without cyclophosphamide was not obviously different, indicating that immunosuppression has mild or no effect on HUCBSC differentiation in rats. Moreover, a few AFP or human ALB positively conjugated nuclear cells were found in rat

liver tissue of HUCBSC-treated group, suggesting that cell fusion and cell division may occur in conjugated nuclear cells.

In conclusion, HUCBSC can differentiate into liver cells *in vitro* and *in vivo* under hepatogenic conditions. Stem cells from UCB are able to differentiate into functional hepatocyte-like cells and may serve as a cell source of cell therapy and transplantation for intractable liver diseases.

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CLINICAL RESEARCH

Predicting utility of a model for end stage liver disease in alcoholic liver disease

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Abstract

AIM: To validate the statistic utility of both the Maddrey Discriminant Function score and the Model for End-Stage Liver Disease as predictors of short term (30 d and 90 d) mortality in patients with alcoholic hepatitis and to assess prognostic factors among clinical characteristics and laboratory variables of patients with alcoholic hepatitis.

METHODS: Thirty-four patients with the diagnosis of alcoholic hepatitis admitted to Hippokraton University Hospital of Athens from 2000 to 2005 were assessed in the current retrospective study and a statistical analysis was conducted.

RESULTS: 30- and 90-d mortality rates were reported at 5.9% (2/34) and 14.7% (5/34), respectively. Significant correlation was demonstrated for the Model for End-Stage Liver Disease ($P_{30} = 0.094$, $P_{90} = 0.046$) and the Maddrey Discriminant Function score ($P_{30} = 0.033$, $P_{90} = 0.038$) with 30- and 90-d mortality whereas a significant association was also established for alanine aminotransferase ($P = 0.057$), fibrin degradation products ($P = 0.048$) and C-reactive protein ($P = 0.067$) with 90-d mortality. For 30-d mortality the Area Under the Curve was 0.969 (95%CI: 0.902-1.036, $P = 0.028$) for the Model for End-Stage Liver Disease score and 0.984 (95%CI: 0.942-1.027, $P = 0.023$) for the Maddrey Discriminant Function score with the optimal cut off point of 30.5 (sensitivity 1, specificity 0.937) and 108.68 (sensitivity 1, specificity 0.969), respectively. Accordingly, for 90-d mortality the Area Under the Curve was 0.762 (95%CI: 0.559-0.965, $P = 0.065$) for the Model for End-Stage Liver Disease score and 0.752 (95%CI: 0.465-1.038, $P = 0.076$) for the Maddrey Discriminant Function score with the optimal cut off point of 19 (sensitivity 0.6, specificity 0.6) and 92 (sensitivity 0.6, specificity 0.946), respectively. The observed Kaplan Meier survival rates

for different score-categories were compared with log-rank tests and higher score values were correlated with a lower survival.

CONCLUSION: Equivalency of the Model for End-Stage Liver Disease and the Maddrey Discriminant Function score is implied by the current study, verified by the plotted Receiver Operative Curves and the estimated survival rates. A statistically significant utility of C-reactive protein, fibrin degradation products and alanine aminotransferase as independent predictors of 90-d mortality has also been verified.

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Key words: Alcoholic liver disease; Alcoholic hepatitis; Maddrey discriminant function score; Model for end-stage liver disease score

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INTRODUCTION

Alcoholic hepatitis (AH) is an acute or acute-on-chronic inflammatory hepatic syndrome manifesting as a result of severe alcohol consumption and correlating with increased mortality rates^[1]. Assessing the severity of the disease is essential for the stratification of patients in need of aggressive therapeutic intervention including corticosteroids and pentoxifylline. A disease severity index for such a purpose should not only have statistical and clinical validity but should preferably rely on a few, readily available, objective parameters and be generalizable to a heterogeneous group of patients^[2].

In 1964 Child and Turcotte introduced the first classification index modified to assess prognosis in patients with severe liver disease. In 1972 Pugh improved that first classification in line with the criticisms of Conn^[3]. The CTP classification is based on serum albumin, serum bilirubin, prothrombin time, ascites and encephalopathy. Although its statistical accuracy has not been methodically evaluated, CTP classification is used as a disease severity

index to determine priority in organ allocation.

The Maddrey Discriminant Function (DF = $4.6 * [PT_{\text{sec}} - \text{control } PT_{\text{sec}}] + \text{serum total bilirubin}_{\text{mg/dL}}$) was introduced in 1978 as a predictor of significant mortality risk in patients with AH and need for aggressive therapeutic intervention. A DF score of greater than 32 identified those patients who had greater than 50% mortality in 30 d outcome^[4,5].

The Model For End-Stage Liver Disease (MELDscore = $3.8 * \log_e[\text{total bilirubin, mg/dL}] + 11.2 * \log_e[\text{INR}] + 9.6 * \log_e[\text{creatinine, mg/dL}]$) was derived from a heterogeneous group of patients from 4 medical centres in the United States and validated in an independent data set from the Netherlands to assess short term survival in cirrhotic patients undergoing elective Transjugular Intrahepatic Portosystemic Shunt (TIPS)^[6]. In the MELD score variables are expressed as logarithm values to avoid extreme values, creatinine is co-evaluated and PT is expressed as an INR which does not depend on the sensitivity of the thromboplastin used by the laboratory.

The aim of the current study is to validate the statistic utility of both DF and MELD scores as predictors of short term (30-d and 90-d) mortality in patients with AH and to assess prognostic factors from among clinical characteristics and laboratory variables of patients with alcoholic hepatitis.

MATERIALS AND METHODS

Materials

Thirty four patients with the diagnosis of AH admitted to Hippokraton University Hospital of Athens between January 1, 2000 and April 30, 2005 were assessed in the current retrospective study. The patients were diagnosed with AH based on the following clinical characteristics: (1) total bilirubin > 1.5 mg/dL, (2) aspartate/alanine aminotransferase ratio above 1.5 with an aspartate aminotransferase level above 45 U/L, (3) alcohol consumption within 2 mo exceeding 40 g/d for male and 20 g/d for female patients and finally (4) absence of a coexistent primary cause of liver disease^[7]. Patients with preexisting viral hepatitis were not excluded from the study on the basis that regeneration of the viral infection could not be established nor an acute viral hepatitis verified as the cause of hospital admission. Survival at 30 and 90 d following hospital admission was verified by chart review or telephone follow-up.

Only laboratory values obtained within 24 h of admission were utilized for calculation purposes. In those patients presenting several hospital admissions only the initial episode was included. The probability of 90-d mortality was calibrated to $P = e^{(-4.3+0.16 * \text{MELD})} / [1 + e^{(-4.3+0.16 * \text{MELD})}]$ ^[7]. The epidemiological data included age, gender, history of alcohol consumption and days of abstinence. Several clinical characteristics and laboratory values were evaluated as independent prognostic variables including fever, corticosteroids or diuretic treatment, infection, hemoglobin, mean corpuscular volume, platelets' count, white blood cell count, spurrcells, aminotransferases, alkaline phosphatase, gamma-glutamyl transpeptidase, bilirubin, creatinine, C-reactive protein, erythrocyte sedimentation rate, α -fetoprotein, prothrombin time, international normalized ratio, fibrinogen, d-dimers, fibrin degradation products,

albumin, ammonia. Clinical features of decompensated hepatic disease including ascites, encephalopathy and edema and also the presence of jaundice were reviewed from the admission history charts. Diagnosis of ascites was based on ultrasound findings and diagnosis of a coexisting infection was established by a positive culture. Hepatic encephalopathy was verified after exclusion of space occupying intracranial lesions, concurrent metabolic, endocrine, traumatic or epileptiform disorders and alcoholic or drug intoxication. Regarding diuretics only those patients receiving diuretics before their hospital admission were evaluated as positive. Non sufficient data was demonstrated regarding the patients' history of alcohol consumption or alcohol abstinence resulting in a request for more detailed documentation of such information in the future.

We searched the database PubMed (1995-2005) using the following key-words: "alcoholic hepatitis", "MELD score", "DF score", "prognosis in alcoholic hepatitis". We also included review articles, book chapters, or commonly referenced older publications. We reviewed the reference lists of articles identified by the search strategy and selected those we judged relevant. The search was restricted to papers published in English.

Methods

Data were analysed using SPSS 11.0 for Windows. Descriptive statistics including mean, median, ranges and standard. Deviation values were calculated for all the continuous baseline demographic, clinical and laboratory characteristics. Univariate logistic regression (backward elimination) was used to screen the variables for statistically significant association with respect to 30- and 90-d mortality. Variables that were statistically significant formed a pool of potential independent predictors. Multivariable logistic regression (backward elimination variables selection procedure) was performed for those variables. The significant factors were kept in the model if the maximum likelihood ratio criterion had a *P*-value below 0.10. Prognostic utility of the different scores was determined by generating a receiver operating characteristic curve (ROC curve). Concordance (range 0.0-1) is equivalent to the area under the curve (AUC) and quantifies the prognostic validities of the variables. Excellent diagnostic accuracy is indicated by AUC between 0.8-0.9 and a c-statistic greater than 0.7 is generally considered a useful test. From the ROC curve coordinates, cut-off points with best sensitivity and specificity of the different scores were determined and predictive values, likelihood and odds ratios were calculated. Overall survival was estimated from the admission data of the patient to the hospital to the date of last follow up or until the patient's death. Kaplan Meier method was used to calculate median follow up and survival curves while the log rank test was used to compare time to events distributions with respect to MELD and DF categories. The death incidences in correlation with MELD and DF values were displayed on scatter plot diagrams.

RESULTS

Thirty four patients with a median age of 49 (\pm 7.74, 9 female and 25 male patients) who met the inclusion cri-

Table 1 Demographics and laboratory values

	N ¹	Range	Mean	Median	Std. deviation
Age	33	36.00-62.00	48.3939	49	7.7498
Yrs of drink	23	3.00-45.00	17.0000	15	10.3177
Alc g/d	22	50.00-360.00	157.7273	150	84.3565
DF	34	15.30-180.72	55.5650	42.25	38.0920
MELD	34	3.00-46.00	20.9118	19	8.5293
Hb	34	5.30-14.80	10.7088	10.85	2.3296
MCV	33	69.10-121.20	100.8606	102.3	11.6788
WBC	34	3000-27000	11300.0000	9340	6039.8068
PLT	34	31000-446000	171970.5882	150000	103840.6370
PT	34	13.10-40.00	20.0044	18.25	6.1619
INR	34	1.10-3.88	1.7415	1.6	0.5922
FIBR/GEN	30	50.00-1350.00	312.6700	289	237.3435
ALBUMIN	32	1.50-4.00	3.0500	3.05	0.4819
SGOT	34	33.00-970.00	164.4412	127.5	165.1286
SGPT	34	19.00-787.00	84.9706	46.5	132.8972
γGT	32	23.00-1967.00	534.7813	353.5	532.8706
ALP	32	65.00-561.00	196.4375	152.5	129.0354
TOTAL BIL	34	1.50-51.00	17.7841	14.73	13.5573
CREAT.	34	0.20-2.80	1.0353	0.95	0.5098
NH3	25	4.84-235.00	127.3936	1.5	60.5252
ESR	28	6.00-141.00	69.0357	72.5	41.7253
CRP	25	1.40-126.00	45.8164	38	40.0220

¹N = number of patients with known laboratory values, yrs: years, alc: alcohol consumption, Hb: hemoglobin, WBC: white blood count, PLT: platelets, PT: prothrombin time, INR: international normalized ratio, SGOT: aspartate aminotransferase, SGPT: alanine aminotransferase, γGT: γ glutamyl transpeptidase, ALP: alkaline phosphatase, BIL: bilirubin, CREAT: creatinine, ESR: erythrocyte sedimentation rate, CRP: C-reactive protein, FIBR/GEN: fibrinogen.

teria were enrolled in the patients cohort. Patients were assessed for a median follow up period of 13 mo (Accrual: February 2000-April 2005; range, 0.6-61.8+; 95%CI: 2.5-23.4). Demographical, clinical and laboratory data were reviewed and mean, median values and ranges are presented (Table 1). Median admission MELD and DF values were 19 (range, 3-46) and 42.25 (range, 15.3-180.72), respectively. 30-d mortality was reported at approximately 5.9% (2/34) whereas 90-d mortality was calculated at 14.7% (5/34). Clinical features of decompensated hepatic disease including ascites, encephalopathy and edema were reported in 64.7%, 32.4% and 52.9% of the patients, respectively. Jaundice was documented in 94.1% of the patients whereas infection was verified in 20.6% of the patients. The presence of serum IgG antibodies indicating an underlying hepatitis A or hepatitis B viral infection was reported in 11.8% (4/24) and 14.7% (5/24) of the patients respectively whereas there was no laboratory confirmation of active viral hepatic infection.

The variables that were significantly associated with 30- and 90-d mortality in univariate logistic regression analysis are presented (Table 2). Significant correlation was demonstrated for MELD ($P_{30} = 0.094$, $P_{90} = 0.046$) and DF scores ($P_{30} = 0.033$, $P_{90} = 0.038$) with 30- and 90-d mortality whereas a significant association was also established for alanine aminotransferase, SGPT ($P = 0.057$), fibrin degradation products, FS ($P = 0.048$) and C-reactive protein, CRP ($P = 0.067$) with 90-d mortality. Notable variables that were not independently correlated with mortality in univariable logistic regression analysis included encephalopathy, ascites, jaundice and albumin.

Table 2 Univariate and multivariate logistic regression assessing 30 d and 90 d mortality

Univariate logistic regression	Variable	P value	Odds ratio	95.0%CI
30-d mortality	DF	0.033	1.051	1.004-1.101
	MELD	0.094	1.357	0.950-1.939
90-d mortality	DF	0.038	1.026	1.001-1.050
	MELD	0.046	1.132	1.002-1.278
	FS	0.048	0.059	0.004-0.979
	SGPT	0.057	7.199	0.944-54.931
	CRP (< 100)	0.910	0.001	0.000-53.00
	CRP (> 100)	0.067	14.000	0.83-235.080
Multivariate logistic regression				
30-d mortality ¹	Step 1 DF	0.484	1.034	0.942-1.134
	MELD	0.707	1.120	0.621-2.021
90-d mortality ²	Step 2 DF	0.033	1.051	1.004-1.101
	Step 5 DF	0.994	12.488	0.00-1.91+297
	MELD	0.997	0.129	0.000

¹30-d mortality: Variable(s) entered on step 1: DF, MELD, variable(s) entered on step 2: DF; ²90-d mortality: Variable(s) entered on step 1: DF, MELD, FS, SGPT, variable(s) entered on step 5: MELD. Only the statistically significant variables are documented.

A statistically significant association couldn't be verified for components that comprise the MELD and DF scores (creatinine, total bilirubin and INR). Variables that exhibited a significant correlation in univariate evaluation were thereafter entered in a multivariate logistic regression process. No additional variables increased the predictive accuracy of either MELD or DF score. In fact all the variables lost significance when they were co-evaluated in a backward variable selection procedure.

MELD and DF scores were plotted in correlation with 30- and 90-d mortality (Figures 1 and 2). Visual inspection of these plots demonstrates that higher MELD and DF values are correlated with an increased death incidence.

Receiver operating characteristics curves were generated in order to validate the predictive accuracy of different scores in assessing 30- and 90-d mortality (Figures 3 and 4). For 30-d mortality the AUC was 0.969 (95%CI: 0.902-1.036, $P = 0.028$) for the MELD score and 0.984 (95%CI: 0.942-1.027, $P = 0.023$) for the DF with the optimal cut off points of 30.5 (sensitivity 1, specificity 0.937) and 108.68 (sensitivity 1, specificity 0.969), respectively. Accordingly for 90-d mortality the AUC was 0.762 (95%CI: 0.559-0.965, $P = 0.065$) for the MELD score and 0.752 (95%CI: 0.465-1.038, $P = 0.076$) for the DF score with the optimal cut off points of 19 (sensitivity 0.6, specificity 0.6) and 92 (sensitivity 0.6, specificity 0.946), respectively.

Kaplan Meier survival rates were estimated for Maddrey score values < 108, ≥ 108 ($P = 0.0098$) and ≥ 92 , < 92 ($P = 0.0002$) and MELD scores ≥ 30.5 , < 30.5 ($P = 0.027$) and ≥ 19 , < 19 ($P = 0.084$). The observed survival rates for different score-categories were compared with log-rank tests and higher score values were correlated with a lower survival. (Figures 5-8)

Prognostic equivalency of MELD and DF scores is implied by the current study, verified by the plotted ROC curves and the cumulative survival curves. A statistically significant utility of CRP, FS and SGPT as independent predictors of 90-d mortality has also been verified.

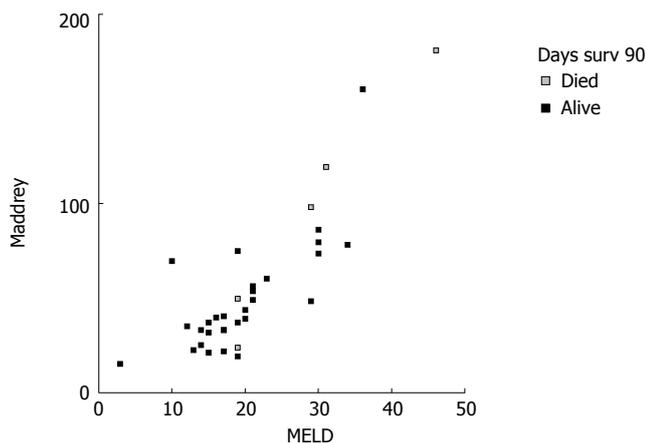


Figure 1 Scatter plot graphed for patient death events within 90 d in correlation with corresponding MELD and DF values.

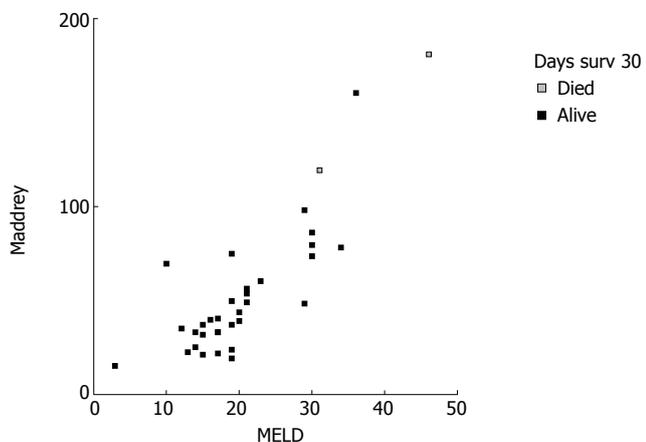


Figure 2 Scatter plot graphed for patient death events within 30 d in correlation with corresponding MELD and DF values.

DISCUSSION

Alcoholic liver disease (ALD) encompasses a clinicohistological spectrum of abnormalities ranging from fatty liver, to AH and irreversible liver cirrhosis (Laennec’s cirrhosis). The potentially fatal clinicopathological syndrome of AH develops in a minority of patients^[9,10].

The major focus of management in AH is abstinence from alcohol, supportive care, treatment of clinical features of decompensated hepatic disease and maintenance of positive nitrogen balance through nutritional support. Although controversy is documented regarding therapeutic issues it is generally agreed that patients with mild disease need not be treated beyond general supportive and symptomatic care and patients with severe disease in extremis may be too ill to correspond in any form of therapy. Identification of those patients who might benefit from aggressive intervention, including corticosteroids or controversial treatment approaches (antioxidant therapy, stimulation of liver regeneration, supplemental amino-acids, inhibition of tumour necrosis factor α and stimulation of collagen degradation^[1]) as well as patients in whom the therapeutic benefit/risk ratio is unfavourable, is currently an issue of great clinical interest.

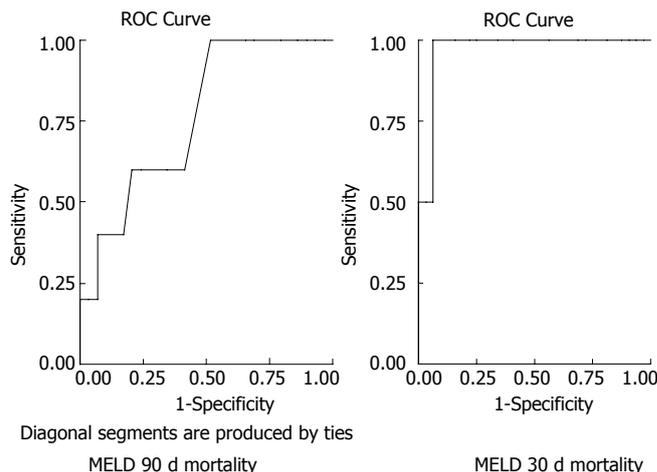


Figure 3 Predicting utility of MELD score in assessing 30- and 90-d mortality rates in alcoholic hepatitis. Receiver operating characteristic curves were generated and the area under the curve and confidence intervals are indicated.

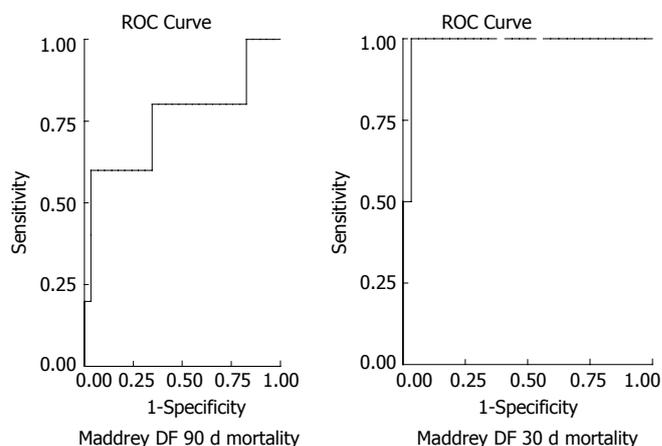


Figure 4 Predicting utility of DF score in assessing 30- and 90-d mortality rates in alcoholic hepatitis. Receiver operating characteristic curves were generated and the area under the curve and confidence intervals are indicated.

On that ground the utility of prognostic models in predicting the short-term mortality in AH has been recently assessed by three series in the literature. In 2002 Sheth *et al* verified the fact that the MELD score performs as well as the DF score in predicting 30-d mortality in AH. A MELD score of greater than 11 or the presence of both ascites and an elevated bilirubin greater than 8 mg/dL should prompt consideration of aggressive therapeutic interventions such as corticosteroids or pentoxifylline according to the authors^[11]. Three years later a retro prospective cohort study assessing 73 patients was conducted by Dunn *et al*^[7], which identified a MELD score of 21 as having the highest sensitivity and specificity for predicting mortality with an estimated 90-d mortality of 20% for patients with this score also manifesting in ascites and encephalopathy. Recommendations were made for such patients to receive aggressive therapeutic agents. According to the authors MELD score maintained some practical and statistical advantages over DF in predicting mortality rates in these patients. Finally in the latest clinical trial conducted in a large cohort of 202 patients with AH, admission, first week and first week change in the MELD score were justified

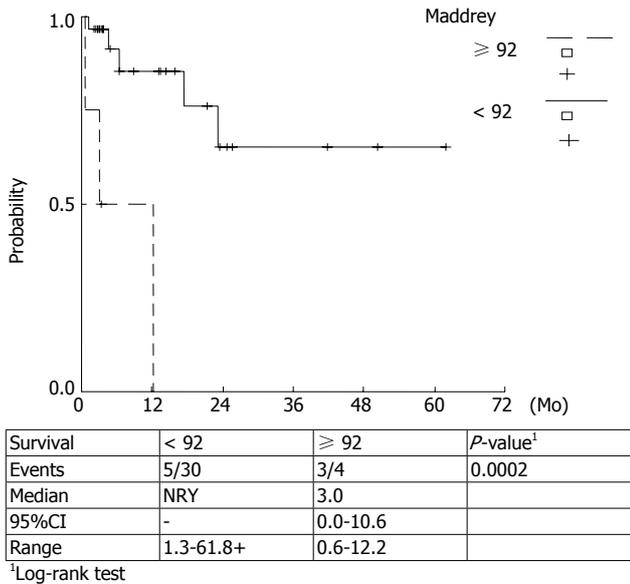


Figure 5 Kaplan Meier survival curves estimated for DF values < 92 and ≥ 92. Higher score values were correlated with a lower survival (P-value 0.0002).

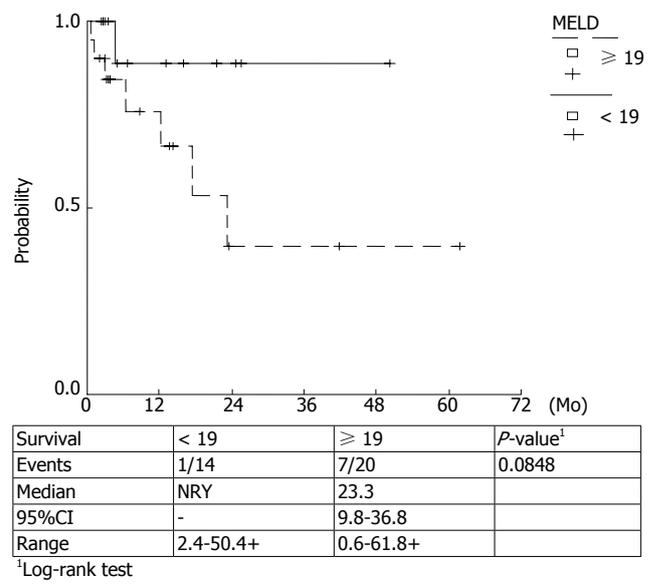


Figure 7 Kaplan Meier survival curves estimated for MELD values < 19 and ≥ 19. Higher score values were estimated to be correlated with a lower survival (P-value 0.0848).

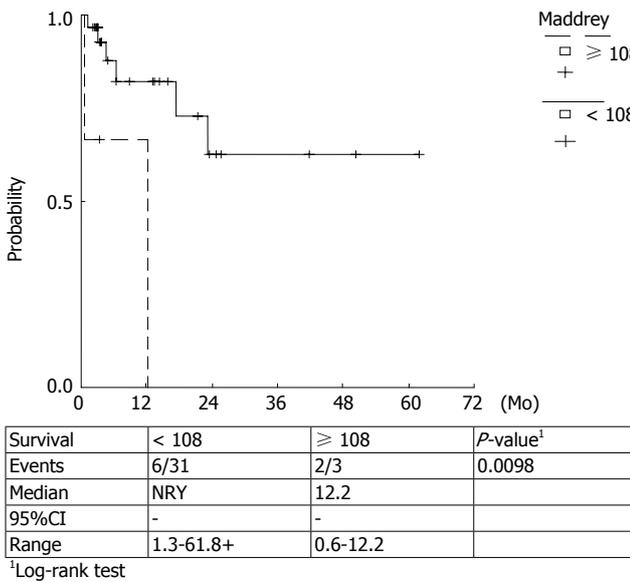


Figure 6 Kaplan Meier survival curves estimated for DF values < 108 and ≥ 108. Higher score values were correlated with a lower survival (P-value 0.0098).

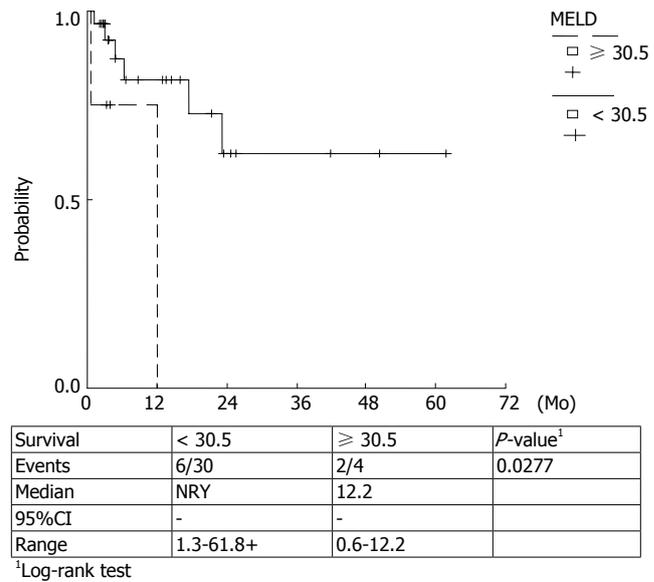


Figure 8 Kaplan Meier survival curves estimated for MELD values < 30.5 and ≥ 30.5. Higher score values were correlated with a lower survival (P-value 0.0277).

as independent predictors for in-hospital mortality. Also the MELD score outranged the DF and CTP scores when considering prognostic accuracy and cut off points of ≥ 18 for admission MELD score, ≥ 20 for the first week MELD score and ≥ 2 for the first week change in MELD score which were significantly correlated with mortality^[8].

In our study a prognostic equivalency of MELD and DF scores was verified whereas the predictive utility of ascites or encephalopathy could not be established. On the other hand, a statistically significant utility of CRP, FS and SGPT as independent predictors of 90-d mortality has been demonstrated. No additional variables significantly changed the prognostic utility of the MELD or DF scores when they were entered in a multivariable analysis. The cut off points were 19 and 30.5 for MELD score and 92 and

108.68 for DF score for 90- and 30- d mortality, respectively.

Despite statistical analysis some practical points favouring the use of the MELD score in this setting should be considered. When compared with the Child-Pugh score the MELD score surpasses in the setting in that: (1) it uses objective parameters which are not subject to center-to-center variability, (2) it increases as the three constituent parameters deteriorate, whereas the individual scoring elements in the Child score remain fixed once a defined threshold has been reached^[12]. Presumably CTP classification is an instrument of its time and implementation of the newest therapeutic strategies will require a more refined scale that accurately represents disease severity. Disadvantages were also demonstrated regarding the utility of the DF classification

for AH including: the use of PT, a variable that is poorly standardized across different laboratories, an established risk of death of up to 17% in patients with a DF score greater than 32^[13,14], and the fact that initial validation of DF correlation to mortality rates is based on series from several decades ago^[7].

In summary, physicians should keep in mind that ALD when complicated by AH should be considered with skepticism and aggressive therapeutic options should be regarded. On that basis prognostic scores should be assessed with a MELD score dominating and presenting sufficient prognostic accuracy.

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Histologic characteristics of gastric polyps in Korea: Emphasis on discrepancy between endoscopic forceps biopsy and endoscopic mucosal resection specimen

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Abstract

AIM: To investigate histological characteristics of gastric polyps in the Korean population.

METHODS: We reviewed endoscopic photographs and medical records of patients with gastric polyps who underwent endoscopic mucosal resection from April 1996 through February 2003.

RESULTS: A total of 85 gastric polyps from 74 patients were reviewed. Male-to-female ratio was 1:1.96. Mean age was 59.9 ± 10.8 years. Multiple polyps were observed in 10.8%. Gastric polyps occurred most frequently in the antrum (58.8%). Pathological results on resected specimens were as follows: tubular adenoma 45.9%, hyperplastic polyp 31.8%, inflammatory polyp 9.4%, hamartoma 3.5%, fundic gland polyp 2.4%, tubulovillous adenoma 2.4%, adenocarcinoma 2.4%, dysplasia 1.1%, and mucosal pseudolipomatosis 1.1%. Discrepancy rate between endoscopic biopsy and pathology of resected specimens was 27.1%. There was no relationship between the size of the polyp and concordance rate.

CONCLUSION: There is considerable discrepancy in histologic findings between endoscopic forceps biopsy and resected specimens. Approaches to review of the histology of an entire polyp should be performed, especially when an adenoma is suspected.

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INTRODUCTION

A gastric polyp is a discrete lesion protruding to the gastric lumen. In autopsy series, the prevalence of gastric polyps is 0.12%-0.8%. However, due to widespread utilization of flexible endoscopy, the frequency of gastric polyps is increasing^[1]. The majority of gastric polyps are adenomatous, hyperplastic and fundic gland polyps, the latter two being the most common^[2-9]. While adenomatous polyps are considered true neoplasms with malignant transformation rates ranging from 6 to 47%^[6,10-13], no such transformations are reported in fundic gland polyps^[6,14]. Although rare, malignant transformations in hyperplastic polyps have been reported^[15,16]. There are reports of discrepancy between endoscopic forceps biopsy (EFB) specimens and endoscopic mucosal resection (EMR) specimens^[17]. Once an adenomatous gastric polyp is found, it is recommended to completely remove the entire polyp to confirm the diagnosis and to remove precancerous lesions. We conducted this study to find out histologic characteristics of gastric polyps in Korean population with an emphasis on the discrepancy in the results of EFB and EMR specimens.

MATERIALS AND METHODS

Patients

We retrospectively analyzed medical records of the patients who underwent EMR of gastric polyps from April 1996 to February 2003 at Seoul Municipal Boramae Hospital. Patients without biopsy results prior to EMR were excluded.

Endoscopic procedures

In the presence of gastric polyps, the size of the polyps was measured using biopsy forceps (open size = 6 mm in diameter). Two to 4 biopsy specimens were taken from each polyp. They were fixed in formalin and sent to the pathologists for further investigation. EMR was performed on a different occasion. Resection margins were marked with a needle knife. Saline was injected submucosally to lift the diseased mucosa off the muscularis propria. After grasping the lesion with forceps, resection was done with a snare that was placed around the lesion.

Histological procedures

EMR specimens were compared with the previous biopsy specimens by a single pathologist. Sections were assessed according to the WHO classification of gastrointestinal tumors published in 1990.

Statistical analysis

Statistical analysis was done using SPSS-PC 11.0 (Statistical package for the social sciences, SPSS Inc., Chicago, IL, USA) for MS Windows®. Data were expressed as mean \pm SD. Categorical data were compared using the χ^2 test. Two-tailed *P* values < 0.05 were considered statistically significant.

RESULTS

Patient characteristics

A total of 74 patients were enrolled to this study. Twenty-five patients were male (a male : female ratio of 1:1.96). Mean age was 59.9 ± 10.8 years (Table 1).

Number of polyps

Sixty-six patients had one polyp (89.2%), 2 patients had 2 polyps (6.8%), and 3 patients had 3 polyps (4.0%). A total of 85 polyps were identified in 74 patients (Table 1).

Location of polyps

Fifty polyps were located in the antrum, 16 in the low body, 9 in the midbody, 5 in the high body, 3 in the angle, and 2 in the fundus (Table 1).

Diameter of polyps

Thirty-six polyps (42.4%) were < 1 cm in diameter, 38 (44.7%) were 1-1.9 cm in diameter, 11 (12.9%) were 2-3 cm in diameter (Table 1).

Classification of polyps according to Yamada classification

Thirty-three (38.8%) polyps were Yamada type I, 24 (28.2%) were Yamada type II, 12 (14.1%) were Yamada type III, and 16 (18.9%) were Yamada type IV (Table 1).

Results of endoscopic forceps biopsy (EFB)

Forty-one (48.2%) polyps were tubular adenoma, 26 (30.6%) were hyperplastic polyps, 13 (15.3%) were inflammatory polyps, 2 (2.4%) were fundic gland polyps, 2 (2.4%) were dysplasia, and 1 (1.1%) was tubulovillous adenoma (Table 1).

Table 1 Patient characteristics, polyp characteristics, and biopsy results

Age (Mean \pm SD, yr)	59.9 \pm 10.8
Sex (Male : Female)	25:49
Number of polyps	
1	66 (89.2%)
2	5 (6.8%)
3	3 (4.0%)
Location of polyps	
Antrum	50 (58.8%)
Body	
Low	16 (18.8%)
Mid	9 (10.6%)
High	5 (5.9%)
Angle	3 (3.5%)
Fundus	2 (2.4%)
Diameter of polyps (cm)	
< 1	36 (42.4%)
1-1.9	38 (44.7%)
2-3	11 (12.9%)
Yamada type	
I	33 (33.8%)
II	24 (28.2%)
III	12 (14.1%)
IV	16 (18.9%)
Endoscopic biopsy results	
Tubular adenoma	41 (48.2%)
Hyperplastic polyp	26 (30.6%)
Inflammatory polyp	13 (15.3%)
Fundic gland polyp	2 (2.4%)
Dysplasia	2 (2.4%)
Tubulovillous adenoma	1 (1.1%)

Table 2 Pathological results of resected specimen

Pathology	n (%)
Tubular adenoma	39 (45.9)
Hyperplastic polyp	27 (31.8)
Inflammatory polyp	8 (9.4)
Hamartomatous polyp	3 (3.5)
Fundic gland polyp	2 (2.4)
Tubulovillous adenoma	2 (2.4)
Adenocarcinoma	2 (2.4)
Dysplasia	1 (1.1)
Mucosal pseudolipomatosis	1 (1.1)
Total	85 (100)

Pathology of EMR specimens (Table 2)

Pathology of EMR specimens were as follows: tubular adenoma, 39 (45.9%); hyperplastic polyp, 27 (31.8%); inflammatory polyp, 8 (9.4%); hamartoma, 3 (3.5%); fundic gland polyp, 2 (2.4%), tubulovillous adenoma, 2 (2.4%); adenocarcinoma, 2 (2.4%); dysplasia, 1 (1.1%); and mucosal pseudolipomatosis, 1 (1.1%). Twenty-four polyps from 18 patients were positive for *H. pylori*. Of 24 polyps, 13 were hyperplastic polyps, 5 were tubular adenoma, 2 were inflammatory polyps, 1 was

Table 3 Histological comparison between results of endoscopic biopsy and resected specimen

Endoscopic biopsy	Resected specimen									Total
	Tubular adenoma	Hyperplastic polyp	Inflammatory polyp	Hamartoma	Fundic gland polyp	Tubulovillous adenoma	Adenocarcinoma	Dysplasia	MP ¹	
Tubular adenoma	35 (85.4%)	0	2 (4.9%)	0	0	1 (2.4%)	2 (4.9%)	1 (2.4%)	0	41
Hyperplastic polyp	1 (3.8%)	22 (84.8%)	1 (3.8%)	1 (3.8%)	0	0	0	0	1 (3.8%)	26
Inflammatory polyp	2 (15.4%)	5 (38.5%)	4 (30.7%)	0	2 (15.4%)	0	0	0	0	13
Fundic gland polyp	0	0	0	2 (100%)	0	0	0	0	0	2
Dysplasia	1 (50%)	0	1 (50%)	0	0	0	0	0	0	2
Tubulovillous adenoma	0	0	0	0	0	1 (100%)	0	0	0	1
Total	39	27	8	3	2	2	2	1	1	85

¹MP, Mucosal pseudolipomatosis

hamartoma, and 1 was tubulovillous adenoma. When hyperplastic polyps with positive *H pylori* were compared with other polyps positive with *H pylori*, hyperplastic polyps showed a correlation with *H pylori* infection ($P = 0.005$).

Comparison of histology between EFB and EMR specimens (Table 3)

Of 41 polyps diagnosed as tubular adenoma in EFB, 35 (85.4%) were tubular adenoma, 2 (4.9%) were inflammatory polyps, 1 (2.4%) was tubulovillous adenoma, 2 (4.9%) were adenocarcinoma, and 1 (2.4%) was a dysplasia. Four cases (9.8%, 1 tubulovillous adenoma, 2 adenocarcinoma, and 1 dysplasia) were underdiagnosed in EFB group when compared with EMR specimens. Two cases (4.9%, inflammatory polyps) were overdiagnosed in EFB as against EMR specimens.

Of 26 polyps diagnosed as hyperplastic polyps in EFB, 1 (3.8%) was a tubular adenoma, 22 (84.8%) were hyperplastic polyps, 1 (3.8%) was an inflammatory polyp, 1 (3.8%) was a hamartoma, and 1 (3.8%) was a mucosal pseudolipomatosis. One case (3.8%, tubulovillous adenoma) was underdiagnosed in EFB compared with EMR specimen.

Of 13 polyps diagnosed as inflammatory polyps in EFB, 2 (15.4%) were tubular adenoma, 5 (38.5%) were hyperplastic polyps, 4 (30.7%) were inflammatory polyps, and 2 (15.4%) were fundic gland polyps. Two cases (15.4%, tubular adenoma) were underdiagnosed in EFB compared with EMR specimens.

Two polyps diagnosed as fundic gland polyps in EFB were proven to be hamartomas in EMR specimen. Two polyps diagnosed as dysplasia in EFB were found to be a tubular adenoma and an inflammatory polyp, respectively. One polyp diagnosed as a tubulovillous adenoma was found to be a tubulovillous adenoma in EMR.

Concordance rate between EFB and EMR was 72.9%. When stratified according to the diameter of polyps, concordance rate was 66.7% (24/36) in polyps < 1 cm in diameter, 78.9% (30/38) in polyps 1-1.9 cm in diameter, 72.7% (8/13) in polyps 2-3 cm in diameter. The concordance rate was not associated with polyp diameter ($P > 0.05$). Concordance rate was not associated with Yamada type or with *H pylori* infection ($P > 0.05$).

DISCUSSION

Gastric polyps are found in less than 1% of the general population^[4]. The rate of malignant transformations in this polyp is less than 1%, usually occurring in polyps with a diameter larger than 1 cm^[15]. Most polyps that undergo malignant transformations are adenomatous polyps. It has been reported that about 11% of adenomatous polyps progress to carcinoma in situ within 4 years of detection^[13].

Contrary to previous reports^[2-9], adenomatous polyps were most frequently encountered in our study. Although this may be due to selection bias, the high frequency of adenomatous polyps (> 40%) in our series imply racial difference in gastric polyp histology.

Discrepancy between EFB and EMR specimen has been reported, with rates ranging from 10 to 25%^[18-20]. In our study, the discrepancy rate between EFB and EMR specimen was 27.1%. Of interest, 2 cases which were diagnosed as tubular adenoma in EFB were later proven to be adenocarcinoma in EMR specimen. Fujiwara and colleagues^[21] reported that 14 of 50 borderline gastric adenomas were diagnosed as containing adenocarcinoma after EMR and that adenocarcinoma could not be detected despite a repeated EFB in 9 patients. In our study, 4 of 41 polyps (9.8%) diagnosed as tubular adenoma were underdiagnosed. This suggests that EFB specimens may not be representative of the entire lesion. Therefore, to obtain a final diagnosis and as well as definitive treatment, lesions should be completely resected by EMR.

It may be expected that as the size of gastric polyp decreases, the biopsy specimen will be more representative of the entire lesion. This was not the case in our study. Although not reaching statistical significances, we found greater discrepancy in smaller polyps.

H pylori infection is reported to be associated with the development of hyperplastic polyps^[8,22]. In our study, hyperplastic polyps were associated with *H pylori* infection. However, *H pylori* infection was not associated with the diagnostic discrepancy observed.

In conclusion, results of our observation of EFB and EMR specimens of gastric polyps showed a certain degree of discrepancy. The size of polyps was not associated with the diagnostic discrepancy. Therefore, especially when adenoma is suspected, evaluation of entire polyp by EMR is warranted regardless of size, to obtain an accurate diagnosis and management plan.

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RAPID COMMUNICATION

A pilot randomized control study to evaluate endoscopic resection using a ligation device for rectal carcinoid tumors

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<http://www.wjgnet.com/1007-9327/12/4026.asp>

Abstract

AIM: Rectal carcinoid tumors smaller than 10 mm can be resected with local excision using endoscopy. In order to remove rectal carcinoid tumors completely, we evaluated endoscopic mucosal resection with a ligation device in this pilot control randomized study.

METHODS: Fifteen patients were diagnosed with rectal carcinoid tumor (less than 10 mm) in our hospital from 1993 to 2002. There were 9 males and 6 females, with a mean age 61.5 years (range, 34-77 years). The patients had no complaints of carcinoid syndrome symptoms. Fifteen patients were randomly divided into 2 groups: 7 carcinoid tumors were treated by conventional endoscopic resection, and 8 carcinoid tumors were treated by endoscopic resection using a ligation device.

RESULTS: All rectal carcinoid tumors were located at the middle to distal rectum. The size of the tumors varied from 3 mm to 10 mm and background characteristics of the patients were not different in the two groups. The rate of complete removal of carcinoid tumors using a ligation device (100%, 8/8) was significantly higher than that of conventional endoscopic resection (57.1%, 4/7). The three patients had tumor involvement of deep margin, for which additional treatment was performed. No complications occurred during or after endoscopic resection using a ligation device. All patients in the both groups were alive during the 3-year observation period.

CONCLUSION: Endoscopic resection using a ligation device is a useful and safe method for resection of small rectal carcinoid tumors.

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INTRODUCTION

It is widely accepted that rectal carcinoid tumors smaller than 10 mm can be treated with local excision using endoscopy because they rarely metastasize^[1,2]. Complete resection of rectal carcinoid tumors is, however, not easy with conventional endoscopic resection methods, as most of these tumors are located in the submucosal layer of the rectal wall of the lower portion of the rectum. As a result, there is a high incidence of residual tumor or tumor near the resection margin, requiring additional surgical intervention^[3,4].

A previous study demonstrated that endoscopic mucosal resection with a ligation device facilitated complete resection of early gastric carcinoma and adenoma^[5]. We previously suggested the possibility of this method for complete resection of rectal carcinoid tumor^[6]. The historical control study performed by Ono *et al*^[4] indicated usefulness of this method for complete resection of rectal carcinoid tumor. In this pilot randomized control trial, we aimed to compare clinical usefulness of ligation device for carcinoid resection with that of conventional endoscopic resection with snaring, and to evaluate a 3-year survival after resection.

MATERIALS AND METHODS

Fifteen patients diagnosed as rectal carcinoid tumor (less than 10 mm) in our hospital from 1993 to 2002 were enrolled in this study. No patients had symptoms of carcinoid syndrome. Examination with a high-frequency ultrasonographic probe (EU-M20; 20MHz, Olympus, Tokyo, Japan) inserted through the biopsy channel of the endoscope was performed for evaluation of carcinoid tumors remained in submucosal layer (Figure 1). After

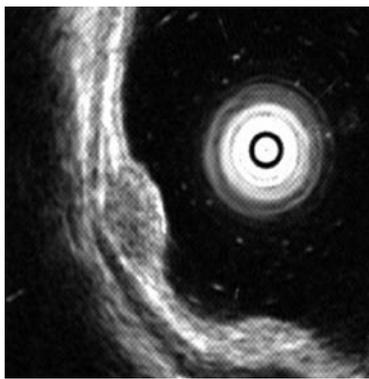


Figure 1 Endoscopic ultrasonography demonstrating a hypoechoic solid tumor in the superficial submucosa without involvement of the muscularis propria.

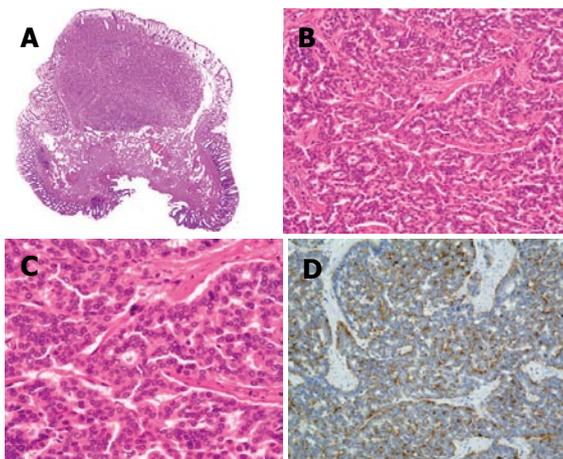


Figure 2 Endoscopic appearance of a carcinoid tumor, 6 mm in diameter, located in the lower portion of the rectum. **A:** Yellowish appearance with a smooth surface before treatment; **B:** injection of submucosal saline solution into the base of the lesion using needle forceps; **C:** aspiration of a carcinoid tumor into the ligation device; **D:** snare resection performed below the band by using blend electrosurgical current.

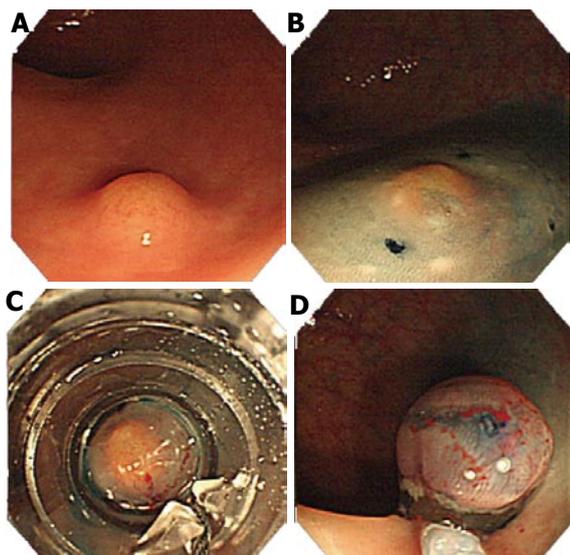


Figure 3 Section of a rectal carcinoid tumor obtained by endoscopic mucosal resection. **A:** Low-power photomicrographs demonstrating a carcinoid tumor, which is present in both the mucosa and submucosa of the rectum, with tumor-free surgical margins (HE, original magnification x 4); **B** and **C:** tumor cells arranged in nests and rosette-like structures, with absence of nuclear pleomorphism and mitotic figures (HE, original magnification x 200 and x 400, respectively); and **D:** chromogranin staining of the tumor cells demonstrating prominent chromogranin immunoreactivity (original magnification x 200).

Table 1 Clinical characteristics of groups 1 and 2 patients

Age (yr)	Sex	Size (mm)	Margin	Additional therapy
Group 1				
74	M	3	Negative	(-)
34	F	5	Negative	(-)
61	M	7	Negative	(-)
46	F	8	Positive	Transanal resection
64	M	6	Negative	(-)
75	M	6	Positive	Ligation
Group 2				
55	M	5	Negative	(-)
77	M	9	Negative	(-)
70	M	7	Negative	(-)
51	F	4	Negative	(-)
63	M	10	Negative	(-)
74	F	4	Negative	(-)
60	F	5	Negative	(-)
51	M	6	Negative	(-)

informed consent was obtained, these patients were randomly assigned into two groups according to a table of random permutations. Group 1 ($n = 7$) carcinoid tumors were treated with endoscopic mucosal resection using a snare with a conventional single-channel colonoscopy. Briefly, after injection of submucosal saline, the lesion was simply resected with snaring. Group 2 ($n = 8$) carcinoid tumors were treated by endoscopic resection using a ligation device (varioligator kit; TOP Co. Ltd, Tokyo, Japan), with modification of the method used in gastric carcinoma^[4,5]. A ligation device attached to a conventional single-channel colonoscopy (PCF200, PCF240 Olympus, Co., Tokyo, Japan), and endoscopic resection using a ligation device was performed as follows: (1) submucosal saline solution was injected beneath the tumor to elevate it for reducing a risk of perforation and resection margin involvement; (2) the carcinoid tumor was aspirated into the ligation device; (3) snare resection was performed below the band by using blend electrosurgical current (Figure 2). This study was performed according to the guidelines of the Committee on Clinical Practice of our hospital.

All resected specimens were examined microscopically for histopathological diagnosis, cut (lateral) and deep (vertical) margin involvement, and vascular and/or lymphatic invasion (Figure 3). Completeness of resection was evaluated by the histopathological examination as absence of carcinoid in the margin of the resected specimen.

Statistical analysis

Tested groups were evaluated using chi-square test or student's *t* test. *P* values of 0.05 or less were considered statistically significant.

RESULTS

The results are shown in Table 1. Average age of group 1 patients was 60.2 years (range: 34-75 years), and average age of group 2 was 62.6 years (range: 51-77

years). Female/male ratios were 3:4 in group 1 and 3:5 in group 2. Thirteen tumors were located in the lower and 2 in the upper portion of the rectum, as 9 sessile and 6 semipedunculated small protrusions of the mucosa. The size of the tumors varied from 3 mm to 9 mm with mean diameter of 6.3 mm in group 1, and from 4 mm to 10 mm with mean diameter of 6.2 mm in group 2. Neither vascular nor lymphatic invasion was observed in any resected carcinoid tumor. Background characteristics of patients were not different between groups 1 and 2.

Complete resection rate was compared in two groups with histopathological examination. The rate of complete removal of carcinoid tumor in group 1 with simple snaring resection was 42.9% (3/7), which was significantly lower compared to that in group 2 with resection using a ligation device (100%, 8/8; $P = 0.024$). The three patients had tumor involvement of deep margin without lateral resection margin. These patients of carcinoid tumor in group 1 were treated with additional treatment: one patient was treated by trans-anal resection and two patients were resected with a ligation device. All patients were followed up more than 3 years, and recurrence of carcinoid tumors was not observed in any patient enrolled in this study.

DISCUSSION

Rectum is the most common part of carcinoid tumors in the gastro-intestinal tract in Japan^[7]. Prevalence of rectal carcinoid has been reported as 0.07% (16/21 522) in healthy subjects^[3] and 0.10% (8/7 665) in subjects of total colonoscopy in Japan^[8]. Most of rectal carcinoid tumors are less than 10 mm in size, and these tumors remain in the rectum without metastasis and can be treated by local resection^[1,2]. These carcinoid tumors are mainly located in the lower part of rectum as shown in this study^[3,8-10].

Rectal carcinoid tumors are treated by several methods including endoscopic resection^[11-13] and surgical operation with trans-anal approach^[14]. Carcinoid tumors less than 10 mm can be treated by local resection, but most of carcinoid tumors extend into the submucosa, and polypectomy can not remove these tumors completely as shown in the previous study^[4]. Complete resection of submucosal tumors requires other method than simple polypectomy. Endoscopic mucosal resection following submucosal injection of saline solution should be useful for the resection.

This randomized control study compared endoscopic resection with a ligation device to conventional endoscopic resection with a snare just after saline injection. As a result, rectal carcinoid tumors less than 10 mm could be removed completely by endoscopic resection with a ligation device without any procedure-related complications. This result confirmed the previous historical control trial performed

by Ono *et al*^[4] who showed usefulness of endoscopic resection with a ligation device but their follow-up period was limited (median follow-up period: 10.5 mo). In this study, we followed up all patients for 3 years without any recurrence.

In conclusion, endoscopic resection with a ligation device might be a most applicable procedure for rectal carcinoid tumors less than 10 mm remained within submucosal layer, because this procedure is simple, minimally invasive, and safe for resection.

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A study on *p53* gene alterations in esophageal squamous cell carcinoma and their correlation to common dietary risk factors among population of the Kashmir valley

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Abstract

AIM: To systematically examine the extent of correlation of risk factors, such as age, consumed dietary habit and familial predisposition with somatic *Tp53* molecular lesion causal to elevate carcinogenesis severity of esophageal squamous cell carcinoma (ESCC) among the Kashmiri population of Northern India.

METHODS: All cases ($n = 51$) and controls ($n = 150$) were permanent residents of the Kashmir valley. Genetic alterations were determined in exons 5-8 of *Tp53* tumor suppressor gene among 45 ESCC cases histologically confirmed by PCR-SSCP analysis. Data for individual cancer cases ($n = 45$) and inpatient controls ($n = 150$) with non-cancer disease included information on family history of cancer, thirty prevailing common dietary risk factors along with patient's age group. Correlation of genetic lesion in *p53* exons to anamistic data from these parameters was generated by Chi-square test to all 45 histologically confirmed ESCC cases along with healthy controls.

RESULTS: Thirty-five of 45 (77.8%) histologically characterized tumor samples had analogous somatic mutation as opposed to 1 of 45 normal sample obtained from adjacent region from the same patient showed germline mutation. The SSCP analysis demonstrated that most common *p53* gene alterations were found in exon 6 (77.7%), that did not correlate with the age of the individual and clinicopathological parameters but showed significant concordance ($P < 0.05$) with familial history of cancer (CD = 58), suggesting germline predisposition at an unknown locus, and dietary habit of consuming locally grown *Brassica* vegetable "Hakh" (CD = 19.5), red chillies (CD = 20.2), hot salty soda tea (CD = 2.37) and local baked bread (CD = 1.1).

CONCLUSION: Our study suggests that somatic chromosomal mutations, especially in exon 6 of *Tp53* gene, among esophageal cancer patients of an ethnically homogenous population of Kashmir valley are closely related to continued exposure to various common dietary risk factors, especially hot salty tea, meat, baked bread and "Hakh", that are rich in nitrosoamines and familial cancer history.

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Key words: Case-controls; Esophageal squamous cell carcinoma; Dietary carcinogens; *p53* alterations

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INTRODUCTION

Among human cancer, esophageal carcinogenesis appears to be a complex multi-step process with a multi-factorial etiology, where environmental, geographical, and genetic factors appear to play major roles^[1]. Apparently 400 000 new cases of esophageal cancer occur each year, and esophageal cancer as such is the sixth leading cause of

cancer death worldwide^[2]. Extended epidemiological studies in high incidence areas, such as Northern Iran, Northern China and South Africa, provide evidence that exposure to specific diet-related nitroso compounds in parallel with nutritional deficiencies and consuming food contaminated by mycotoxins are the most important determinants of the disease^[3]. Also in some parts of India, esophageal cancer is alarmingly rising and is as such the third leading cancer in men and fourth leading cancer in women in these regions^[4]. Reports from Southern India suggest that esophageal squamous cell carcinoma (ESCC) occurs in more than 80% of cases in chronic tobacco smokers that is further potentiated by heavy use of alcohol and additional prevailing risk factors, including nutritional factors and vitamin deficiencies^[5]. In the Kashmir valley, belonging to the Northern part of India, esophageal cancer has been reported to exceed 40% of all cancers, however, very few reports have associated this malignancy with specific risk factors prevalent in the area^[6].

With the advent of molecular biology, new strategies are being carried out for the prevention and treatment of cancer. Cumulating evidence indicates that changes in both dominant oncogene and tumor suppressor genes are likely for malignant transformation of normal cell^[7]. Among these genetic abnormalities, *p53* tumor suppressor gene, a critical regulator of cell growth, differentiation and apoptosis, is frequently affected in most human cancer^[8]. The frequencies of *Tp53* alterations in esophageal carcinoma vary from 26% to 87% that occur in multiple sites throughout the open reading frame, which are mostly limited to DNA binding domain that spans exon 5 through exon 8^[9]. The current study was initiated to find out the risk factors of esophageal cancer in Kashmiri inhabitants and to correlate *p53* alterations in ESCC with prevalent risk factors.

MATERIALS AND METHODS

Collection of tumor samples and data from case-control population

Hospital and general population-based case-control study of permanent inhabitants of Kashmir was performed between March 2003 and December 2005. Cases were selected from the Government Medical College, Srinagar, Kashmir and controls from three districts of Kashmir valley, India viz. Srinagar, Anantnag and Pulwama. From each of the three districts, 150 healthy individual families (controls) were randomly selected and evaluated along with selected patients on a detailed questionnaire for demographic information as well as exposure to etiological factors, including dietary nitrosoamines, intake of pickled vegetables, meat, hot salty tea, source of water, *etc* as well as the incidence of any family history of cancer in them. Family history of cancer was defined as the presence of esophageal cancer in one or more relatives for both cases and controls. One hundred milligram of tumor specimen and other 100 mg of macroscopically normal adjacent esophageal tissues were obtained from each selected ESCC patient (cases) that were admitted in different Wards of Government Medical College, Srinagar, Kashmir and were diagnosed for the first time for such type of cancer. The selected cases diagnosed for the first time for ESCC

having no chemotherapy or radiotherapy prior to biopsies were interviewed 3 d prior to the surgery. From 51 histopathologically confirmed ESCC samples, information related to histopathological grade *etc* was collected from the Division of Pathology, Government Medical College, Srinagar. For evaluating the association between the common dietary risk factors and genesis of ESCC in Kashmiri inhabitants, the dietary habits of all the 45 cases were compared with two groups of controls (Group II and Group III), each representing 45 normal subjects taken from total of 150 healthy individual families. Group II represented the controls with family history of cancer and group III represented the controls without any family history of cancer.

DNA extraction and PCR amplification

High molecular genomic DNA from the fresh ESCC and from adjacent normal tissue samples was extracted using DNAzol reagent (Imperial Bio-Medics, India). The extracted DNA was used for PCR amplification reactions. PCR primers used were same as used previously for the amplification of 5-8 exons of *TP53* gene^[19]. Out of total 51 ESCC cases, 6 with apparent cancer of other origin were excluded from PCR amplification and only 45 were used for the further study. All the reactions were carried out in a total volume of 50 μ L in Techgene (0.2 mL) PCR System (Techne, UK). Typical PCR conditions were as follows: denaturation at 94°C for 10 min, followed by 35 amplification cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. An elongation step at 72°C for 10 min was added to the final cycle for all the exons. PCR products were separated on 40 g/L agarose gel continuously and visualized by 600 g/L ethidium bromide staining.

SSCP analysis

Non-radioactive SSCP was performed as described previously^[10] with slight modification. A 60 g/L non-denaturing gel conditions were used with 60 mL/L glycerol (dissolved in 0.5 \times TBE buffer). Aliquots of amplified products were mixed with equal amounts of denaturing buffer (500 mL/L deionized formamide and 500 mL/L glycerol with 0.5 g/L xylene cynol and 0.5 g/L bromophenol blue). Samples were denatured at 95°C for 5 min, kept on ice until loading on the 60 g/L non-denaturing gel and run in vertical electrophoretic plates (Banglore Genei, India) with 0.5 \times TBE as running buffer at 150 V for 3-4 h at room temperature. SSCP fragments were visualized by silver staining.

Statistical analysis

Analysis of the data was performed on the original data by using chi-square (χ^2) test to determine correlation between *p53* alterations in ESCC and dietary risk factors as well as with clinicopathological status of ESCC tissue samples. $P < 0.05$ was considered statistical significant.

RESULTS

Samples, clinicopathological parameters and *p53* mutations

Forty-five cases without any apparent cancer of other

Table 1 Correlation between clinicopathologic findings and *p53* genetic alterations (*n* = 45)

Factors	patients <i>n</i> (%)	<i>p53</i> alterations		¹ <i>P</i> value
		Positive (%)	Negative (%)	
Age (yr)				0.848
< 60	10 (22.2)	8 (80)	2 (20)	
> 60	35 (77.7)	27 (77.14)	8 (22.8)	
T-status				0.128
T1	5 (11.1)	3 (60)	2 (40)	
T2	7 (15.5)	3 (42.8)	4 (57.1)	
T3	22 (48.8)	19 (86.3)	3 (13.6)	
T4	11 (24.4)	8 (72.7)	3 (27.2)	
N-status				0.114
N0	15 (33.3)	10 (66.6)	5 (33.3)	
N1	30 (66.6)	26 (86.6.3)	4 (13.3)	
M-status				0.2
M0	25 (55.5)	18 (70)	7 (30)	
M1	20 (44)	15 (75)	5 (25)	
Cell-differentiation				0.21
Well		25 (75.7)	8 (24.2)	
Moderate		3 (42.8)	4 (57.1)	
Poor		3 (60)	2 (40)	

¹Chi-square (χ^2) test with Fisher's exact test was used to test the frequency distribution of *p53* alteration.

origin, and 150 inpatient controls were enrolled in the current study, among which most of the cases (77.8%, 35/45) were older than 60 years (Table 1). Thirty-three of 45 (73.3%) tissue samples were histologically confirmed to be well-differentiated, 15.5% (7/45) moderately differentiated and 11.1% (5/45) poorly differentiated cancer. Regarding depth of tumor invasion, 5 (11.1%) cases belonged to T1, 7 (15.5%) cases belonged to T2, 22 (48.8%) cases belonged to T3 and 11 (24.4%) cases belonged to T4. Our results demonstrated no difference in the incidence of *p53* alterations among the patients below and above 55 years. With respect to *p53* alterations and depth of tumor invasion, T3 and T4 demonstrated higher frequency of mutations (> 72%) as compared to T1 and T2. However, statically no positive correlation was found between tumorous *p53* alterations and age ($P = 0.848$), depth of tumor (T) invasion ($P = 0.128$), lymph node metastasis (M) ($P = 0.114$) and cell differentiation ($P = 0.210$) (Table 1).

Risk factors of esophageal cancer

In the general population (controls)-based survey representing 50 individual families, each from three districts of Kashmir valley, matched for different life styles and dietary habits revealed some common food habits that were also common in the selected ESCC cancer patients (Table 2). Total 51 ESCC cases, 45 cases without cancer of any other origin and represented by Group I showed 35 (77.7%) cases with family history of esophageal cancer. Two groups of controls were used representing 51 families, with one group designated as Group II showing the family history of cancer and Group III without any family history of cancer. Interestingly, more than 80% subjects in Group I and Group II showed the same pattern of dietary habits i.e. heavy consumption of hot salty tea,

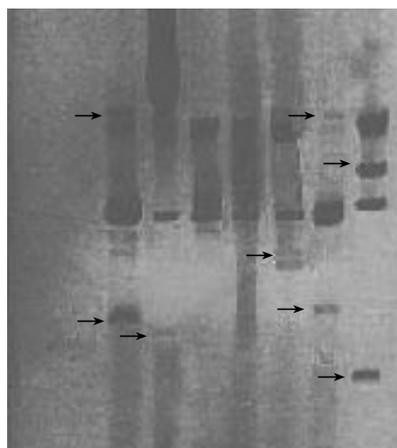


Figure 1 Single-strand conformational polymorphism (SSCP) of *p53* exon 6 in ESCC specimens showing 8 anomalous bands with altered mobility and indicates the presence of *p53* mutation (arrows).

local baked bread, meat, red chillies as well as consumed "Hakh", a locally grown green leafy vegetable belonging to the *Brassica* family, very heavily (0.57 kg/person per wk) as compared to subjects in group III. Statistically, we also could not find any remarkable difference quantity-wise in consumption of Hakh, hot salty tea, baked bread, meat and red chillies between the group I and Group II. However, both of these two groups differed significantly from the Group III with respect to consumption of these aforementioned food items at very lower amounts (Table 2).

Correlation of *p53* alterations in ESCC with the prevalent risk factors

Interestingly, 32 of 35 (91.42%) tumor samples from selected 45 cases (Group I) with family history of cancer showed positive for *p53* alterations as compared to only 3 of 10 (30%) samples from cases without any family history of cancer. On SSCP analysis, a total of 82 anomalous bands from these tumor DNA of 35 ESCC patients in exons 5-8 with single exon and multiple exon alterations were observed (Table 3). One case also showed an anomalous band in DNA from both normal constitutional DNA as well as tumor DNA. Statistically, the genetic alterations in 5-8 exons of *p53* gene in total of 45 cases differer significantly with respect to each other ($\chi^2 = 11.25$, $P = 0.0105$) and most of the mutations (77.7%) were confined to exon 6 (Figure 1), followed by exon 7 (38.9%), exon 8 (33.3%) and exon 5 (27.7%) in decreasing order. Statistically, *p53* alterations, especially in exon 6, were closely related to familial history of cancer (CD = 58), suggesting germline predisposition at an unknown locus, and also to prevalent dietary habit of consuming locally grown *Brassica* vegetable "Hakh" (CD = 19.5), red chillies (CD = 20.2), hot salty soda tea (CD = 2.37) and local baked bread (CD = 1.1).

DISCUSSION

Reports indicate that the Kashmir valley of Jammu and Kashmir State of India ranks among the highest incidence area for esophageal cancer in the world^[6]. Despite the gravity of the problem, very little work has initiated in the area. In the current study, 77.7% patients were older than 60, and thus indicating ESCC to be the malignancy of adults with 80% of them found to seek treatment in the

Table 2 Some specific dietary habits of three groups consisting each of 51 individuals

Dietary group	Group I	Group II	Group III	At <i>P</i> values < 0.05, CD
Baked bread (Kandaroo Roti)	41/51 (5 roti/d per person)	50/51 (4 roti/d per person)	45/51 (2 roti/d per person)	1.1
Hot salty tea with soda	43/51 (5 cups/d per person)	51/51 (4 cups/d per person)	48/51 (2 cups/d per person)	2.37
Meat	48/51 (0.82 kg/wk per person)	51/51 (0.75 kg/wk per person)	48/51 (0.500 kg/wk/person)	0.25
Red chillies	51/51 (78 g/wk per person)	50/51 (90 g/wk per person)	30/51 (38 g/wk per person)	20.2
<i>Brassica oleracea</i> (Hakh)	51/51 (0.35 kg/wk per person)	51/51 (0.57 kg/wk per person)	51/51 (0.55 kg/wk per person)	19.5

advanced stage of their cancer and diagnosed for the first time^[5]. These results indicate the lack of general awareness of this disease among the general population and remain a major health concern that needs immediate medical attention in the area.

The important cause for high incidence of disease in the Kashmir valley may be defined on the basis that unlike Ladakh and Jammu constituencies of Jammu and Kashmir state that are the low incidence area for ESCC, the Kashmiri population on account of climatic conditions practice an exclusively different dietary life-styles, including use of hot salty tea, red chillies, baked bread, meat, high-nitrate diet especially locally grown *Brassica* leaves, the Hakh (Table 2). To our knowledge, scant literature that have been documented till date suggests diet-related *N*-nitroso compounds in parallel with nutritional deficiency to be most important determinants of disease in this area^[11,12]. Our investigation provides evidence that the controls with family history of cancer (group II) followed more or less the same dietary habits as that of patients (Group I), indicating consumption of such types of dietary risk factors rich in nitroso compounds associated with carcinogenesis of ESCC. It is noteworthy that Group II subjects developed much more chance of getting ESCC than subjects from Group III that consumed very low quantity of such foods (Table 2). Thus, in accordance with earlier findings, our results also suggest that there is a chance of higher rate of new esophageal cancer in the population that is exposed to common dietary risk factors than those without such exposures^[13]. Among such dietary risk factors, heavy consumption of hot salty soda tea provides chronic irritation of esophageal epithelium causing predisposition of carcinogenic substances to initiate tissue-specific malignant transformation and at the same is also a rich source of carcinogens like *N*-nitrosoproline and nitrosopipelic acid^[11]. On individual basis, the daily dietary nitrate intake (237 mg) in Kashmir valley is much higher than the values reported for most of the Western countries (i.e., Germany 75 mg/d and Britain 95 mg/d) and that through consumption of "Hakh" alone that seems to have also a great importance as potential source of nitroso compounds and their precursors^[14,15]. Thus, the results clearly indicate that on daily basis, the Kashmiri population gets exposed to high amounts of nitrate, nitrite and precursor of nitroso

Table 3 Frequencies of *p53* genetic alternations detected by PCR-SSCP in esophageal squamous cell carcinoma (*n* = 45)

	<i>p53</i> exons 5-8							
	Exon 5		Exon 6		Exon 7		Exon 8	
	(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)
No. of cases (%)	13	32	35	10	18	27	15	30
	28.8	71.1	77.7	22.2	40	60	33.3	66

compounds that are in high enough quantity to be one of the responsible dietary risk factor for this malignancy in the area^[11,14]. Additionally, high consumption of red chilli pepper in the region also indicates higher risk for them towards gastric cancer than non-consumers^[6]. Thus exposure of Kashmiri inhabitants to such high or even to low levels of these dietary compounds throughout the life could be involved in the carcinogenesis of esophagus in this high-risk area.

Most of the researchers now suggest various food-borne carcinogens associated with the genesis of ESCC through the involvement of *p53* tumor suppressor gene. Such reports thus suggest a molecular epidemiology approach to investigation of cancers for which the causes have remained elusive^[6,17]. In accordance to earlier reports, interestingly, our study could not find any positive association between clinicopathological variables and *p53* alterations in the collected samples^[18] (Table 1). On the other hand, current study, in contradiction to earlier report, demonstrates most of the alterations (14/18) confined to exon 6 of *p53* gene^[1]. These results are in consonance with most of the other earlier reports that showed more than 50% of mutations confined to exon 6 of *p53* gene among selected ESCC cancer patients^[19].

Finally, this preliminary investigation of tumor gene alterations in patients from Kashmir valley thus supports a large body of epidemiological observations, pointing to dietary mutagenic carcinogenesis and family history of cancer peculiar to populations at high risk of esophageal cancer. The results clearly indicate that such somatic alterations of *p53* gene, especially in its exon 6, in ethnically homogenous population of Kashmir valley are closely related to specific dietary habit and family history of cancer^[6]. However, family history of cancer depends on many factors and cannot as such conclude that the positive

familial history of cancer is due to genetic susceptibility. It is finally suggested that the gradual change in dietary and cultural features in the population should produce a significant decrease in esophageal carcinogenesis in the area, and at the same time, in order to draw some logical conclusion, further studies with large set of samples and data are strictly warranted from this part of India.

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RAPID COMMUNICATION

Antiviral treatment of hepatitis B virus-transgenic mice by a marine organism, *Styela plicata*

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Abstract

AIM: To evaluate the antiviral effect of the effective ingredient of *Styela plicata* in a murine model of hepatitis B virus carrier.

METHODS: HBV-transgenic mice were divided into 3 groups (control group, lamivudine treatment group and the effective ingredient of *Styela plicata* treatment group) and assigned to receive normal diet, lamivudine or the effective ingredient of *Styela plicata* for consecutive weeks. Serum hepatitis B surface antigen was detected by enzyme-linked immunosorbent assay (ELISA) method. Serum HBV DNA was detected by real-time polymerase chain reaction (RT-PCR). Serum T helper (h) 1 cytokine interleukin (IL)-2 and Th2 cytokine IL-6 were detected by the quantitative sandwich enzyme immunoassay technique. Another group of HBV-transgenic mice was assigned to receive the effective ingredient of *Styela plicata* for consecutive weeks. The histology of liver tissue was evaluated before and after treatment.

RESULTS: Twelve weeks after starting the therapy, serum hepatitis B surface antigen was significantly lowered in *Styela plicata*-treated mice and lamivudine-treated mice compared with the mice receiving normal diet ($F_{12wk} = 88.81$, $P_{12wk} = 0.000 < 0.01$). Serum HBV DNA was significantly lowered in *Styela plicata*-treated mice and lamivudine-treated mice compared with the mice receiving normal diet ($F_{12wk} = 20.71$, $P_{12wk} = 0.000 < 0.01$). However, like lamivudine, the effective ingredient of *Styela plicata* could not inhibit the replication of HBV completely. A rebound phenomenon of hepatitis B sur-

face antigen and HBV DNA in sera could be found 4 wk after withdrawal of medication. Eight weeks after starting the therapy, serum levels before and after *Styela plicata* treatment of IL-2 were 2.41 ± 0.38 and 10.56 ± 0.78 ng/L, respectively ($t_{8wk} = -16.51$, $P_{8wk} = 0.000 < 0.01$). Compared with the serum levels of IL-2 in the normal diet-treated mice (2.48 ± 0.17 ng/L; $t_{8wk} = 13.23$, $P_{8wk} = 0.000 < 0.01$). Serum levels before and after *Styela plicata* treatment of IL-6 were 63.62 ± 6.31 and 54.52 ± 6.22 ng/L, respectively, compared with the serum levels of IL-6 in the normal diet-treated mice (60.84 ± 4.21 ng/L). Histological analysis of liver from *Styela plicata*-treated HBV-transgenic mice also showed catabatic status in inflammation and hepatitis B surface antigen.

CONCLUSION: *Styela plicata* may be an effective antiviral medicine in treating chronic hepatitis B.

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Key words: *Styela plicata*; Hepatitis B virus; Transgenic mice; RT-PCR; Chronic hepatitis B

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INTRODUCTION

Chronic hepatitis B virus (HBV) infection is a serious clinical problem because of its worldwide distribution and possible adverse sequelae, such as hepatic decompensation, cirrhosis, and hepatocellular carcinoma (HCC)^[1,2]. Although interferon and lamivudine are recommended for patients with chronic liver disease (CLD)^[3,4,6], the high cost, adverse side effects, lower levels of efficacy, and the inability to block the progression to HCC have tremendously limited the use of them^[5-7].

In the absence of an ideal therapy many chemicals with biological activities are used in patients with chronic hepatitis. Ascidians, an intertidal marine, are known for their capacity to inhibit tumors, bacterium and virus^[8-10]. Recent data have indicated that the effective components of the ascidian *Styela plicata* (Sp) can inhibit the secretion of hepatitis B surface antigen (HBsAg) and hepatitis B e

antigen (HBeAg) in 2.2.15 cells^[11].

HBV-transgenic mice (HBV-Tg), which express products of the HBV genome and also show signs of HBV replication, are a suitable animal model to study the process of destruction of hepatocytes, the critical role of antiviral medicines in controlling HBV replication and gene expression^[3,12,13]. In the current study, we evaluated the anti-HBV activity of the alcohol extract of Sp by conducting therapeutic trials in four groups of HBV-Tg expressing similar levels of HBV-markers. A highly significant therapeutic effect of Sp was demonstrated. The mechanism underlying the therapeutic role of Sp in anti-HBV therapy needs to be further investigated.

MATERIALS AND METHODS

Animals

The HBV (adr subtype) transgenic mice (HBV-Tg) were prepared by microinjection of complete 2.0 copy HBV genome into pronuclei of fertilized eggs^[14]. The HBV DNA can be expressed and replicated in the transgenic mice. HBV genomic DNA in mouse line could be transmitted to next generations. The transgenic mice are tolerant to HBV gene products and similar to human chronic HBV carrier^[14,15]. Their serum HBsAg can be expressed highly, but serum HBeAg is expressed inapparently.

We purchased the HBV-Tg of either sex from the Infectious Diseases Center of 458 Hospital of Chinese PLA at 6 wk of age. All animals were housed with controlled temperature at 28°C ± 2°C and 16:8 h light-dark cycle.

Animal grouping

HBV-Tg were divided into three groups. Group 1 (9 mice, the control group) was administered with normal foodstuff and physiological saline. Group 2 (9 mice, a treatment group) was allowed to receive lamivudine (50 mg/kg per day), normal foodstuff and physiological saline. Group 3 (9 mice, a treatment group) was administered with the foodstuff containing the effective ingredient of Sp (5 g/kg per day) and physiological saline. Every group was gastrogavaged for 12 wk. The sera of HBV-Tg were collected before administration of drugs, at the 4th, 8th and 12th wk after administration of drugs, and the 4th wk after withdrawal of drugs.

Another group of HBV-Tg (5 mice) were anesthetized by an injection of amobarbital (70 µg/g). Then an abdominal incision was made. Parts of their livers were immediately excised. Then the incisions were closed. After 2-wk feeding with ordinary foodstuff and physiological saline, the mice were assigned to the foodstuff containing the effective ingredient of Sp (5 g/kg per day) and physiological saline for 10 wk and later the livers were obtained with the method described before.

Assay of hepatitis B surface antigen

HBsAg in sera was detected quantitatively by the enzyme-linked immunosorbent assay (ELISA) method, as described in commercial kits (Rongsheng Biotech Co., Ltd, Shanghai, China). The HBsAg levels were expressed

in optical density (OD) values. All performance strictly followed the manuals of the ELISA kits.

Quantitative detection of serum HBV DNA

For HBV DNA quantification by real-time polymerase chain reaction (RT-PCR), viral DNA was extracted from serum using viral DNA extraction solution (DaAn Gene Co., Ltd, Guangzhou, China) according to the manufacturer's instructions. PCR amplification was performed with a set of PCR primers and a probe corresponding to the surface antigen gene of HBV^[15]. The PCR primers are: P1, 5'-ATCCTGCTGCTATGCCCTCA TCTT; P2, 5'-ACAGTGGGGGAAAGCCCTACGAA. The TaqMan probe is 5'-TGGCTCAGTTTACTAGT-GCCATTTG. The PCRs were performed according to the manufacturer's instructions (DaAn Gene Co., Ltd, Guangzhou, China)^[16] using a LightCyclerTM (Roche Diagnostics, Germany). The PCR cycling program consisted of an initial denaturing step at 93°C for 2 min, followed by 40 amplification cycles at 93°C for 5 s, 57°C for 45 s. The HBV DNA titers > 1.00 × 10³ (1.00E03) copies/mL were considered to be positive.

Detection of serum cytokines

Sera were collected before administration of drugs, at the 4th, 8th wk after drug administration. Levels of IL-2 and IL-6 in sera were detected by using ELISA kits specific for IL-2 and IL-6 (Senxiong Technology Industry Co., Ltd, Shanghai, China). The assay used the quantitative sandwich enzyme immunoassay technique. All operations strictly followed the manuals of the ELISA kits.

Histological analysis

Some of the specimens obtained were fixed with 4% buffered formalin and embedded in paraffin. Then they were stained with haematoxylin and eosin (HE) and examined on a video monitor. The normal and inflamed regions of the specimens were easily discriminated by HE stains. The degree of liver cell degeneration and inflammation was evaluated according to previous method^[17].

The other specimens obtained were fixed with 4% buffered formalin and embedded in paraffin. Then they were stained with orcein and examined on a video monitor. The HBsAg could be observed by orcein stains.

Statistical analysis

The experiment results were analyzed by SPSS12.0 statistical software. The data for the negative rate were expressed as percentage. The data for the concentration of HBV DNA in serum were expressed as mean ± SD. Difference before and after therapy within one group was analyzed using paired *t*-test. Difference among the groups was evaluated using analysis of variance (One-way ANOVA). Statistical significance was accepted at the level of *P* < 0.05.

RESULTS

Changes of HBsAg titers

The changes of HBsAg titers are shown in Table 1. The

Table 1 Changes of HBsAg titers (mean \pm SD)

Treatment group	OD values				
	0 wk	4 wk	8 wk	12 wk	16 wk
Group 1 normal diet recipients (n = 9)	1.61 \pm 0.01	1.61 \pm 0.01	1.62 \pm 0.02	1.62 \pm 0.01	1.60 \pm 0.02
Group 2 lamivudine recipients (n = 9)	1.59 \pm 0.03	1.00 \pm 0.21 ^{a,d}	0.92 \pm 0.15 ^{b,d}	0.76 \pm 0.08 ^{b,d}	1.29 \pm 0.11 ^{a,d}
Group 3 Sp recipients (n = 9)	1.61 \pm 0.02	1.32 \pm 0.08 ^a	1.01 \pm 0.16 ^{b,d}	0.82 \pm 0.07 ^{b,d}	1.05 \pm 0.05 ^{b,d}

^aP < 0.05, ^bP < 0.01 compared with T0 in one group; ^dP < 0.01 compared with group 1.

Table 2 Anti-HBV DNA production (mean \pm SD)

Treatment group	Serum HBV DNA (copies/mL)				
	0 wk	4 wk	8 wk	12 wk	16 wk
Group 1 normal diet recipients (n = 9)	1.35E05 \pm 3.64E04	1.34 E05 \pm 3.20 E04	1.53 E05 \pm 3.10 E04	1.30 E05 \pm 2.43 E04	2.59 E05 \pm 9.37 E04
Group 2 lamivudine recipients (n = 9)	1.20 E05 \pm 2.54 E04	5.52 E04 \pm 1.63 E04 ^b	2.48 E04 \pm 8.12E03 ^{b,d}	1.34 E04 \pm 4.48 E03 ^{b,d}	1.12 E05 \pm 2.70 E04
Group 3 Sp recipients (n = 9)	1.73 E05 \pm 4.69 E04	8.39 E04 \pm 4.32 E04	4.07 E04 \pm 1.63 E04 ^{a,d}	1.19 E04 \pm 7.53 E03 ^{b,d}	1.91 E05 \pm 6.87 E04

^aP < 0.05, ^bP < 0.01 compared with T0 in one group; ^dP < 0.01 compared with group 1.

paired *t*-test showed that the HBV-Tg which responded to lamivudine therapy had reduced serum HBsAg compared with that prior to therapy from wk 4 to wk 12 ($t_{4wk} = 2.94$, $P_{4wk} = 0.039 < 0.05$; $t_{8wk} = 7.37$, $P_{8wk} = 0.000 < 0.01$; $t_{12wk} = 13.54$, $P_{12wk} = 0.000 < 0.01$). The HBV-Tg which responded to the effective ingredient of Sp showed reduced level of serum HBsAg from wk 4 to wk 12 significantly ($t_{4wk} = 2.89$, $P_{4wk} = 0.020 < 0.05$; $t_{8wk} = 3.61$, $P_{8wk} = 0.007 < 0.01$; $t_{12wk} = 11.11$, $P_{12wk} = 0.000 < 0.01$). Although these treatment groups all exhibited a rebound phenomenon 4 wk after withdrawal of medication, they still exhibited a significant lower level of serum HBsAg compared with that prior to therapy ($t_{lami} = 2.39$, $P_{lami} = 0.043 < 0.05$; $t_{sp} = 10.15$, $P_{sp} = 0.000 < 0.01$). One-way ANOVA showed that the HBV-Tg which responded to lamivudine had a significant reduced level of serum HBsAg compared with the control group which responded to normal diet from wk 4 to wk 12 ($F_{4wk} = 5.47$, $P_{4wk} = 0.003 < 0.01$; $F_{8wk} = 11.59$, $P_{8wk} = 0.000 < 0.01$; $F_{12wk} = 88.81$, $P_{12wk} = 0.000 < 0.01$); the HBV-Tg which responded to the effective ingredient of Sp showed a significant reduced level of serum HBsAg compared with the control group which responded to normal diet from wk 8 to wk 12 ($F_{8wk} = 11.59$, $P_{8wk} = 0.001 < 0.01$; $F_{12wk} = 88.81$, $P_{12wk} = 0.000 < 0.01$). Although these treatment groups all exhibited a rebound phenomenon 4 wk after withdrawal of medication, they still exhibited a significant lower level of serum HBsAg compared with the control group which responded to normal diet ($F_{16wk} = 14.79$, $P_{lami} = 0.011 < 0.05$, $P_{sp} = 0.000 < 0.01$).

Anti-HBV DNA production

The anti-HBV DNA production is summarized in Table 2. The paired *t*-test showed that the HBV-Tg which responded to lamivudine therapy had reduced serum HBV DNA compared with the level of HBV DNA prior to therapy from wk 4 to wk 12 ($t_{4wk} = 5.76$, $P_{4wk} = 0.000 <$

0.01 , $t_{8wk} = 4.23$, $P_{8wk} = 0.003 < 0.01$, $t_{12wk} = 4.00$, $P_{12wk} = 0.004 < 0.01$). The HBV-Tg which responded to the effective ingredient of Sp showed a significant reduced level of serum HBV DNA from wk 8 to wk 12 ($t_{8wk} = 2.43$, $P_{8wk} = 0.041 < 0.05$; $t_{12wk} = 3.57$, $P_{12wk} = 0.007 < 0.01$). One-way ANOVA showed that the HBV-Tg which responded to lamivudine had a significant reduced level of serum HBV DNA compared with the control group which responded to normal diet from wk 8 to wk 12 ($F_{8wk} = 11.36$, $P_{8wk} = 0.000 < 0.01$; $F_{12wk} = 20.71$, $P_{12wk} = 0.000 < 0.01$). The HBV-Tg which responded to the effective ingredient of Sp showed a significant reduced level of serum HBV DNA compared with the control group which responded to normal diet from wk 8 to wk 12 ($F_{8wk} = 11.36$, $P_{8wk} = 0.001 < 0.01$; $F_{12wk} = 20.71$, $P_{12wk} = 0.000 < 0.01$). Both the two groups exhibited a rebound phenomenon 4 wk after withdrawal of medication.

Antiviral effect

HBV-Tg receiving lamivudine showed a fast cure rate compared with HBV-Tg receiving the effective ingredient of Sp. However, their long-term effects were similar (Figure 1), HBV-Tg receiving either the effective ingredient of Sp or lamivudine exhibited the same cure rate (33%) 12 wk after the start of therapy. Both groups showed a rebound phenomenon 4 wk after withdrawal of medication.

Changes of cytokine titers

Before treatment, there was no significant difference in the serum levels of IL-2 and IL-6, respectively, between the control group and the treatment group. After weeks of treatment, the paired *t*-test showed that the HBV-Tg which responded to the effective ingredient of Sp therapy increased serum IL-2 compared with the level of IL-2 prior to therapy from wk 4 to wk 8 ($t_{4wk} = -6.74$, $P_{4wk} = 0.000 < 0.01$; $t_{8wk} = -16.51$, $P_{8wk} = 0.000 < 0.01$); and the IL-2 serum

Table 3 Data are expressed as mean \pm SD

Treatment group	IL-2 (ng/L)			IL-6 (ng/L)		
	0 wk	4 wk	8 wk	0 wk	4 wk	8 wk
Group 1 normal diet recipients (<i>n</i> = 9)	2.13 \pm 0.23	2.33 \pm 0.26	2.48 \pm 0.17	63.59 \pm 1.82	65.40 \pm 10.3	60.84 \pm 4.21
Group 3 Sp recipients (<i>n</i> = 9)	2.41 \pm 0.38	7.91 \pm 1.09 ^{b,d}	10.56 \pm 0.78 ^{b,d}	63.62 \pm 6.31	61.80 \pm 4.58	54.52 \pm 6.22

^b*P* < 0.01 vs T0 in one group; ^d*P* < 0.01 vs group 1.

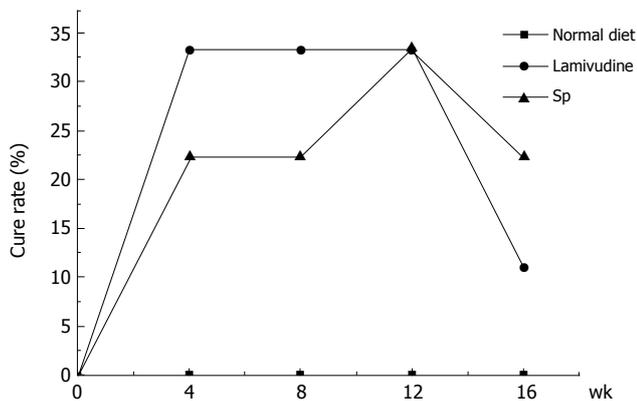


Figure 1 Rate of responses following therapy. Thirty-three percent (3 of 9) of HBV-Tg receiving lamivudine became completely negative for HBV-DNA compared with 22% (2 of 9) of HBV-Tg receiving the effective ingredient of Sp 8 wk after the start of therapy. They had the same cure rate (33%, 3 of 9) 12 wk after the start of therapy. But the inhibitory effect could not last long. A rebound phenomenon could be found 4 wk after withdrawal of medication. HBV-Tg receiving normal diet did not show such therapeutic response.

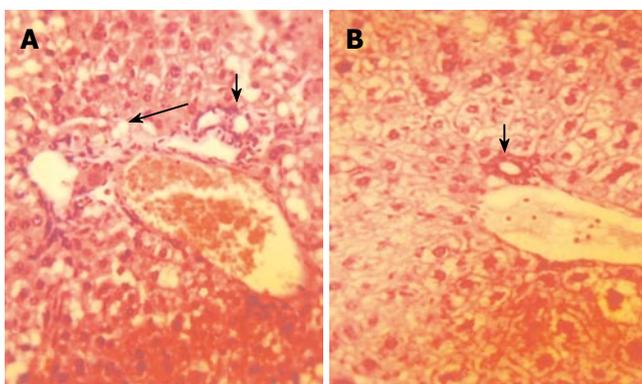


Figure 2 Representative examples of haematoxylin and eosin (HE) staining of HBV-Tg liver (\times 400). The expression of infiltrating lymphocyte was strongly positive (white arrow) before drug administration. The structure of hepatic lobule was obscure and the swelling degree of liver cells was serious. Many vacuoles (black arrow) could be seen in (A). However, these findings were alleviated after 10 wk of therapy by Sp (B).

levels were higher in the Sp-treated group compared with those of the control group ($I_{4wk} = 6.58$, $P_{4wk} = 0.000 < 0.01$; $I_{8wk} = 13.23$, $P_{8wk} = 0.000 < 0.01$). Although a slight decline could be seen, there was no significant difference in the serum levels of IL-6 before and after treatment (Table 3).

Histological observation

HBV-Tg receiving the effective ingredient of Sp for 10 wk exhibited improvement in inflammation and HBsAg in

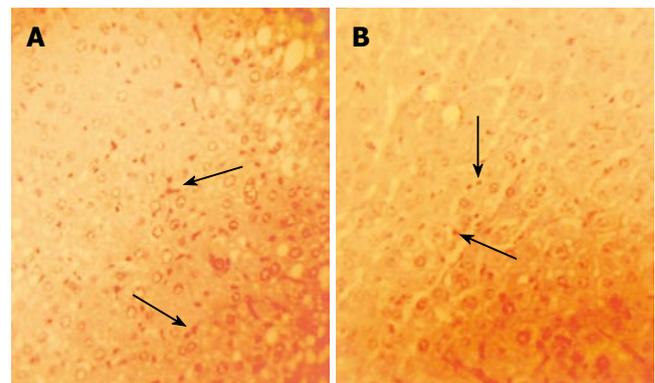


Figure 3 Representative examples of orcein staining of HBV-Tg liver (\times 400). HBsAg was seen as nigger-brown granules before drug administration (A) and after 10 wk of therapy by Sp (B). However, a decrease in the signals for HBsAg was seen in (B) compared with that in (A).

Table 4 Inflammatory degree and degeneration degree of hepatocytes in HBV-Tg liver after 10 wk of treatment (mean \pm SD, *n* = 5)

	0 wk	10 wk
Degeneration degree of hepatocytes	8.00 \pm 0.63	5.60 \pm 0.68 ^b
Inflammation degree of hepatocytes	5.20 \pm 0.37	4.00 \pm 0.32 ^a

^a*P* < 0.05, ^b*P* < 0.01 vs T0 in one group.

liver compared with HBV-Tg receiving normal diet (Figures 2 and 3). The semi-quantitative evaluation of inflammatory degree and hepatocytes degeneration degree in mouse liver proved the above findings (Table 4).

DISCUSSION

Ascidians are marine animals with a high ability to synthesize bioactive substances. The extracts of ascidians are most commonly used in antimicrobial assays and anticancer treatments^[18-20]. Bioactivity-guided ingredientation of an ethyl acetate extract of the marine ascidian, *Pseudodistoma* sp., collected in the Tsitsikamma Marine Reserve, revealed that the antimicrobial properties in this extract resided in a group of acyclic amino alcohols, isolated as their peracetylated derivatives^[18]. The extract of *Eudistoma vancouveri* Millar, obtained from the northeastern Brazilian coast showed a high toxicity in tumor cell lines tested^[20].

Sp is a dominant component of the marine benthos

of South China Sea. In recent years, we carried out basic research on the marine organism. The alcohol extracts of the animal have a definite inhibition effect on HBsAg and HBeAg *in vitro*, although the exact mechanism is not known^[11,21,22].

This study suggested that the effective ingredient of Sp might have potential therapeutic value for chronic hepatitis B infection. Thirty-three of the HBV-Tg receiving Sp responded to the therapy and became completely negative for HBV-DNA 12 wk after the start of therapy (Figure 1). The level of serum HBsAg titers was also reduced, though not significantly. But like most present antiviral drugs, the effective ingredient of Sp could not inhibit the replication of HBV completely. Relapse after cessation of the alcohol extract of Sp could be observed, similar to that of lamivudine (Table 1 and Table 2).

In our experiment, we found that the gait and status of psychosis between the effective ingredient of Sp-treated HBV-Tg and the normal diet-treated ones were similar. Histological analysis of mouse liver revealed that the alcohol extract of Sp could ameliorate the inflammation in liver, alleviate hepatocytes degeneration and eliminate HBsAg (Figures 2 and 3, Table 4). Besides, the histological observation showed that there was no distinct toxicity in tissue. However, we did not have further virological/molecular study of HBV markers in the liver. To find more molecular/pharmacological mechanisms of the effect of Sp, we need to measure the HBV DNA and cccDNA levels, as well as HBsAg and HBeAg expression in liver in the following experiments.

Then, we investigated the effects of the effective ingredient of Sp on the serum levels of Th1/Th2 cytokines. It is well known that the identification of functionally distinct CD4⁺ T helper subpopulations, producing distinct patterns of cytokines, has provided an important insight into the mechanisms by which polarized immune response occurs *in vivo* in response to pathogens^[23]. T helper 1 cytokines, including IFN- γ , IL-2 and TNF- β are involved principally in cell-mediated immunity and play a crucial role in the protection from intracellular pathogens, including a number of viruses, while Th2-derived cytokines (IL-4, IL-5, IL-6 and IL-10) stimulate antibody production and promote mast cell and eosinophil proliferation^[24,25]. The cytokine pattern secreted by T cells on viral antigen recognition is believed to exert a profound influence on both the type of disease caused by the infecting agent and the final outcome of the viral infection. In recent years, researchers have found that the prevalent Th1 pattern of secreted cytokines can be regarded not only as a mechanism contributing to inflammation and local tissue damage, but also as an appropriate response of the immune system to create conditions that hamper viral replication and eventually lead to HBV eradication^[26]. Our experiment results showed that the effective ingredient of Sp could selectively increase the serum level of IL-2 (which is helpful for normal biologic immunity) and prevent the further increase of serum IL-6 (which can promote hepatocytes degeneration) in HBV-Tg (Table 3). These are important clues to therapeutic mechanism of Sp on chronic hepatitis B.

Then, what is the therapeutic mechanism of Sp? It is

still undefined. We suppose the explanations as follows: First, there are many chemical compositions such as peptide, alkaloids, saponin, macrolide, terpenoid in Sp. Coordinated action of them may be the main mechanism. Second, Sp is abundant in polypeptide and polysaccharide. Some compositions of them can stimulate cellular immunity, induce the differentiation of T lymphocyte and the generation of endogenous interferon, kill target cells and inhibit the replication of HBV. Third, mineral substances and vitamins in Sp also play main roles. Mineral substances are important in liver enzyme metabolism. Vitamins participate in multifold metabolism of liver. They can induce the generation of hepatocytes and enhance the detoxification function of liver.

In conclusion, the effective ingredient of *Styela plicata* has a definite effect on chronic hepatitis B. Our data provide valuable information for understanding the therapeutic role and the potential therapeutic mechanism of *Styela plicata* as an effective antiviral medicine in treating chronic hepatitis B.

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RAPID COMMUNICATION

Detection of *H pylori* antibody profile in serum by protein array

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serum samples. It is especially useful for large scale epidemiological investigation of the infection of *H pylori*.

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Key words: *Helicobacter pylori*; Protein array; Antibody; Immunogold

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Abstract

AIM: To detect multiple *H pylori* antibodies in serum samples of individuals who carry *H pylori* by protein array.

METHODS: Recombinant *H pylori* antigens, urease B subunit (UreB), vacuolating toxin A (VacA) and cytotoxin associated gene A protein (CagA), were prepared and immobilized in matrixes on nitrocellulose membrane by robotics to bind the specific immunoglobulin G (IgG) antibodies in serum. Staphylococcus protein A (SPA) labeled by colloid gold was used to integrate the immuno-complex and gave red color signal. The scanner based on charge-coupled device (CCD) could collect the image signal and convert it into digital signal.

RESULTS: When human IgG was printed on the membrane in increasing concentrations and incubated with immunogold, a linear dose response curve was obtained and the detection limit for IgG was about 0.025 ng. The cutoff values, which were defined as the mean grey level plus 3 times of standard deviation, were 27.183, 28.546 and 27.402, for anti-UreB IgG, anti-CagA IgG and anti-VacA IgG, respectively, as 400 human serum samples with negative *H pylori* antibodies were detected by the protein array. When 180 serum samples from patients in hospital were employed for detection of IgG against UreB, CagA and VacA, the sensitivity of the protein array was 93.4%, 95.4%, 96.0%, and the specificity was 94.8%, 94.4% and 97.5%, respectively, as compared with the results obtained by ELISA. The assay also showed high reproducibility, uniformity and stability, and the results were available within 30 min.

CONCLUSION: The protein array is a very practical method for rapid detection of multiple antibodies in

INTRODUCTION

H pylori chronically infect more than half of the world population and are associated with chronic gastritis, peptic ulcer and even gastric cancer^[1-7]. Several virulence factors of *H pylori*, such as urease, vacuolating toxin A (VacA) and cytotoxin associated gene A protein (CagA) have been characterized^[8-11]. Urease is produced by all *H pylori* and functions to hydrolyze urea into CO₂ and NH₃ which can buffer the acid environment and permit the bacterium survival in stomach. All *H pylori* strains carry *vacA* gene and only 50% of them express VacA protein, which assembles into a flower-shaped oligomer, alters intracellular vesicular trafficking and induces vacuole formation in eukaryotic cells. The most important feature that distinguishes *H pylori* strains is the presence or absence of the *cag* pathogenicity island (*cag* PAI). It contains about 30 genes and codes for a type IV secretion machinery system (TFSS), through which CagA is introduced into host cell^[12,13]. Phosphorylated CagA in cytoplasm dephosphorylates host cell protein (cortactin), altering cytoskeletal structure and forming a hummingbird phenotype^[14,15]. CagA is implicated in host cell signal transduction system^[16,17], and CagA positive *H pylori* are much more closely related with peptic ulcer and gastric cancer in western country^[4,6]. When *H pylori* infect human being, multiple antibodies are generated against various antigenic compounds and an antibody library may form in serum. Therefore, screening the antibodies against these virulence factors in serum of *H pylori* infected individual is useful for detection and classification of the pathogen.

Serological assays for diagnosis of *H pylori* infection are included among the noninvasive methods recommended

by the European *H pylori* study group^[18,19]. At present, the common method to detect *H pylori* antibodies in serum is enzyme-linked immunosorbent assay (ELISA). The procedure is time-consuming especially when multiple antibodies are detected at the same time. Although there are reports regarding the detection of multiple proteins in an antibody-based protein microarray system^[20-21], the labor-intensive procedures and the expensive instrumentations have limited its application^[22]. Based on the previous work, we developed a low-cost protein array system for rapid detection of multiple *H pylori* antibodies in serum samples.

MATERIALS AND METHODS

Preparation of antigens

Recombinant urease B subunit (UreB, 40 Ka), N-terminal fragment (amino acid: 1-284, 38 Ka) of CagA and middle region fragment (amino acid: 579-907, 30 Ka) of VacA were produced in our institute previously^[23]. The purity of these proteins was 95%, 96% and 96%, respectively, as identified by coomassie stained gel and evaluated by dual-wave length flying-spot scanner CS-9000 (Shimadzu). The antigenicity was defined as the dilution titer of the antigen to give 1.0 OD in ELISA when the standard positive serum samples (Xinkang Company, Shenzhen) were used. If the original concentration was 1 mg/mL, the dilution titer of UreB, CagA, and VacA was 1:800, 1:1000, and 1:600, respectively.

Preparation of protein array

Nitrocellulose membrane (Pharmacia) with a pore diameter of 0.45 μm was immersed in 0.05 mol/L carbonate buffer (pH 9.0) and dried at room temperature. The antigens (0.1%) in 0.01 mol/L phosphate-buffered saline (PBS, pH 7.0) were transferred from the micro-well plate to the membrane by use of the stainless steel solid pin (0.7 mm in diameter) and dotted on the nitrocellulose membrane by using computer-controlled high speed robotics, MicroGrid II (BioRobotics). The printing was performed in a cabinet at 4°C, with 60% humidity. Each pin was estimated to transfer 10 nL of the solution. The protein array consisted of 4 matrixes, in three of which the antigens were printed in 9 replicates. Another one was the control matrix printed of rabbit myosin (R.M, Sigma) and human IgG (H. IgG, Sigma) (Figure 1A). The membrane was blocked by 5% bovine serum albumin (BSA, Sigma) at 4°C for 2 h and then washed twice by weakly shaking in the wash buffer for 10 min. After drying at room temperature, the membrane was cut into small pieces (1.2 cm \times 1.2 cm) according to the positions properly marked and assembled into the *H pylori* protein array apparatus. The longitudinal section of the protein array apparatus was illustrated in Figure 2.

Preparation of immunogold

Colloid gold was made according to the sodium citrate reduction method^[23]. In brief, 200 mL of 0.01% chloroauric acid (analytical grade) was heated to the boiling point and 8 mL of the 1% sodium citrate (analytical grade) was then added under vigorously stirring condition. When the solution became dark red in color, the boiling was

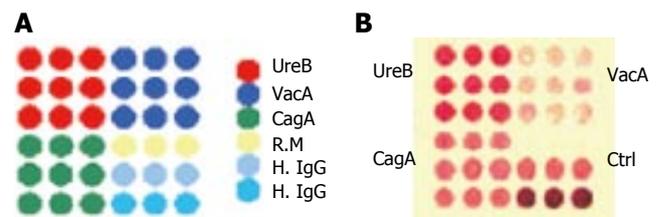


Figure 1 A: Schematic map of protein distribution on the protein array. Four squares were involved and different proteins were printed in different areas: UreB in upper left area; VacA in upper right area; CagA in left bottom area; R.M (10 ng) in the upper row of right bottom area for negative control (Ctrl), H. IgG in the middle row for positive control and at the bottom row for strong positive control; B: positive results of anti-UreB IgG, anti-CagA IgG and anti-VacA IgG detected by the protein array.

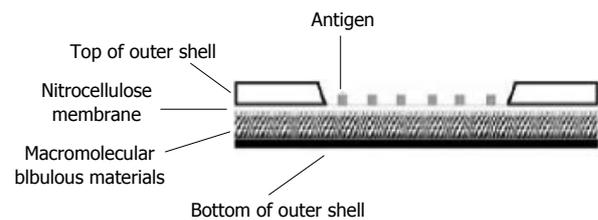


Figure 2 Longitudinal section of the protein array apparatus. The shell of the apparatus was made of plastic materials. A window was opened on the top of the outer shell and the margin of the nitrocellulose membrane printed with antigens was sealed to the inner surface of the window. Under the nitrocellulose membrane was the macromolecular bilbulous material.

lasted further for 5 min. The colloid gold should have the highest light absorbance at 525 nm. The shape and size were observed and the diameter (15 nm) was measured under transmission electron microscope. One hundred microlitres of colloidal gold was taken and the pH value was adjusted to 6.2 by 0.1 mol/L potassium carbonate. Staphylococcus protein A (SPA) was added to the final concentration of 1.5 mg% under stirring condition. Ten minutes later, 10 mL of 5% BSA was added and the colloidal gold was stirred for 5 min, making sure that there was no bubble. The immunogold was centrifuged at 4000 g, 4°C for 5 min. The supernatant was then loaded to the Sephacryl S-400 column (Pharmacia) and eluted with 0.02 mol/L tris-buffered saline (pH 7.4). The main red portion was collected and centrifuged at 12 000 g, 4°C for 10 min. The pellets were then suspended in 100 mL solution (0.01 mol/L PBS, 1% BSA) and stored at -20°C for further use.

Procedures to detect antibodies in serum samples

The procedures are as follows: Firstly, 4 drops of the washing solution were added to the center of the nitrocellulose membrane to immerse it evenly. Secondly, 100 μL of the five times diluted serum was added onto the membrane, and 6 drops of the washing solution were dripped onto it to wash the residual serum 5 min later. Thirdly, 100 μL of the immunogold was added onto the membrane, and again, 6 drops of the washing solution were dripped onto it 5 min later. Finally, the protein array was placed into the scanner, which adopts charge-coupled device (CCD) camera to collect the grey level of the central part (0.5 mm in diameter) of every spot and converts the image signal into digital signal. The results

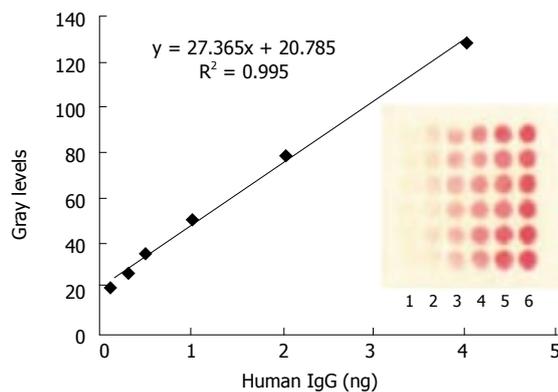


Figure 3 The mean grey level of the spot against various amount of H.IgG immobilized on the protein array. The concentrations of human IgG spotted from lane 1 to lane 6 are as follows: 1.25, 2.5, 5, 10, 20, 40 μ g/mL. The optimized amount of SPA binding to colloidal gold was 12 μ g/mL. The data in the plot were obtained from the image of CCD by averaging the mean grey levels of the 6 replicate spots.

were given according to the positions of the matrixes and the mean grey levels of the spots in the matrixes.

Detection of the antibodies in serum samples

Eight hundred and sixty-five serum samples were collected from blood centers in Xi'an and Shenzhen. *H pylori* antibodies (anti-UreB IgG, anti-CagA IgG and anti-VacA IgG) negative serum samples were screened by using the ELISA kits (kit to detect IgG against *H pylori* CagA, Jingying Company, Shanghai; kits to detect IgG against *H pylori* CagA and VacA, Xingkang Company, Shenzhen). The cutoff values of anti-UreB IgG, anti-CagA IgG and anti-VacA IgG were then determined. One hundred and eighty serum samples, collected from patients with different background in Xi'an Center Hospital, were employed to evaluate the sensitivity and specificity of the protein array in detection of *H pylori* antibodies. Rapid urease test (RUT) and ELISA were also used to detect the infection of *H pylori*.

Statistical analysis

All experiments were performed in replicate. χ^2 and Student's *t* test were used for statistic analysis.

RESULTS

Limit of detection

Human IgG was printed in 6 replicates in increasing concentrations and incubated with 100 μ L of immunogold on nitrocellulose membrane. A linear dose response curve was obtained (Figure 3) and the detection limit for IgG was about 0.025 ng bound on membrane in this system.

Amount of antigen immobilized

Each antigen with different concentrations ranging from 0.1 to 2.0 ng/nL was printed on the membrane to make the protein array. Five serum samples with *H pylori* antibody titer of 1:32 in ELISA were used as references to detect the relevant antibody by protein array. The least amount

Table 1 Cutoff values determined in *H pylori* antibody negative sera

Items	Number	Mean grey values	SD	Cutoff values
Anti-UreB IgG	400	18.060	3.041	27.183
Anti-CagA IgG	400	17.992	3.518	28.546
Anti-VacA IgG	400	17.730	3.224	27.402

of antigen that defined all the samples as positive was designated as the optimized one. In this way, the amount of antigen chosen to immobilize on membrane was 12, 10, and 16 ng for UreB, CagA and VacA, respectively.

Determination of the cutoff values

The cutoff values, which were defined as the mean grey level plus 3 SD, were 27.183, 28.546 and 27.402, for anti-UreB IgG, anti-CagA IgG and anti-VacA IgG, respectively, as 400 serum samples with negative *H pylori* antibodies were detected by the protein array (Table 1).

Uniformity and reproducibility

When a protein array was randomly chosen from the same batch and subjected to detection of the antibodies in a serum sample, the coefficient variation (CV) of the grey values of the 9 spots in every matrix was less than 8%. When 10 protein arrays were randomly chosen to detect the antibodies in a serum sample, the mean grey value of every matrix in every protein array was obtained and the CV of the mean grey values from the ten relevant matrixes was less than 10%. Ten quality control serum samples, 5 positive and 5 negative for the three antibodies detected by ELISA, were used to screen the antibody profiles by protein array. The results were the same when the test was repeated 5 times.

Sensitivity and specificity

For the 180 serum samples collected from clinical patients, the antibodies were screened by both ELISA and protein array. The results showed that, of these samples, 117, 108 and 99 were positive for anti-UreB IgG, anti-CagA IgG and anti-VacA IgG respectively by protein array (positive results of the three antibodies are shown in Figure 1B), and 122, 109 and 101 were positive respectively by ELISA. The sensitivity of protein array in detection of anti-UreB IgG, anti-CagA IgG and anti-VacA IgG was 93.4%, 95.4%, and 96.0%, and the specificity was 94.8%, 94.4% and 97.5% respectively, compared with the results detected by ELISA (Table 2).

Relation of *H pylori* antibody profiles to diseases

The positive rates of anti-UreB IgG in groups of gastric cancer (GC, 26/32) and peptic ulcer (PUD, 39/45) were significantly higher than that in group of non-ulcer dyspepsia (NUD, 52/103) ($P < 0.005$). There was no significant difference in the positive rates of anti-CagA IgG or anti-VacA IgG between different patient groups (Table 3).

Table 2 Results detected by ELISA and protein array

Protein array	ELISA					
	Anti-UreB IgG		Anti-CagA IgG		Anti-VacA IgG	
	P	N	P	N	P	N
P	114	3	104	4	97	2
N	8	55	5	67	4	77

P : positive; N: negative.

DISCUSSION

Biochip technique is an advanced tool that enables the binding of multiple molecules in a small area at one time^[24,25]. The molecules (protein, nucleic acid or amino acid) can be immobilized on a piece of support substance such as glass, silica, plastic, nitrocellulose and even metal. The fixation of the molecules to the support substance is by means of non-specific absorption, affinity absorption or covalent attachment, depending on the purpose to design the biochip. Methods based on protein array by non-specific absorption are successfully used for serodiagnosis of infectious diseases in some laboratories^[26-28]. The protein array in the current investigation was designed in accordance with the recent trends including mini-turization of assays and the simultaneous measurement of a panel of antibodies in a single assay.

Actually, this protein array is an immunoassay based on dot immunogold filtration assay. Nitrocellulose membrane was chosen as the chip and the recombinant antigens were immobilized on it by non-specific adsorption. The membrane can bind more amount of protein compared with the glass surface, as each pore of the membrane can be considered as a column and the antibodies can be accumulated when they pass through the membrane. Moreover, the proteins in the membrane are in three-dimensional structure and the binding of antigen and antibody is highly efficient^[29]. Colloid gold was used to label SPA as it was red in color and did not need any substance to produce signal for detection. The reader, which was CCD based video collection system to convert image signal into digital signal, made the results more objective. The entire system offers the simultaneous determination of several antibodies in one test with low cost and faster kinetic. It takes less than 30 min to finish the whole procedures. The stability was quite good and the specificity and sensitivity were close to those of ELISA when *H pylori* antibody profiles of 180 serum samples were detected. Therefore, it is the most practical means of obtaining accurate and precise results. All these make the method a promising future for application, especially for large scales investigation of *H pylori* infection.

In western countries, about 50% to 60% *H pylori* isolates are cagA positive, and the rates are much higher in Asia-Pacific areas^[30-32]. In this study, the positive rates of anti-UreB IgG in groups of GC and PUD were significantly higher than that in group of NUD, indicating that *H pylori* infection was more closely related with GC and PUD, whereas the role of anti-CagA IgG or anti-VacA IgG in prediction of specific diseases was limited.

Table 3 Relation of diseases to *H pylori* antibody (IgG) profiles

Diseases	Numbers				
	Total	RUT	Anti-UreB	Anti-CagA	Anti-VacA
GC	32	30	26 ^a	25	22
PUD	45	43	39 ^c	37	36
NUD	103	58	52	46	41

^aP < 0.005 vs NUD; ^cP < 0.005 vs NUD.

In the pathogenesis study of the *H pylori*, we know very well that the living conditions of this bacterium *in vivo* and *in vitro* are quite different. Some proteins contributing to virulence of the bacterium may not be expressed under *in vitro* culture conditions, and their expression may depend on certain *in vivo* stimuli, such as, cell to cell contact^[33]. So antibody profile may be a reliable indication to reflect the host immune response to the bacterium. Since two genomes of this bacterium have already been sequenced, functional study of these genes is becoming the most important target and many studies have been carried out in the disease related gene screening and identification^[34-37]. With more *H pylori* pathogenic genes being known, more proteins may be added onto the protein array. Obviously, to optimize the binding condition of antigen and antibody and to select one that is suitable for a panel of reactions are the key to success. By accumulating more data about the clinical background of the patients and the antibody profiles in sera, we can draw a clear picture of the relationship between the two aspects.

In summary, detection of multiple *H pylori* antibodies in serum by protein array has the prospect in clinical application, especially in developing countries or small hospitals. Further studies are still going on to increase the density of the protein array.

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Changes of nitric oxide and endothelin, thromboxane A₂ and prostaglandin in cirrhotic patients undergoing liver transplantation

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Abstract

AIM: To investigate the perioperative changes of nitric oxide (NO) and endothelin (ET), thromboxane A₂ (TXA₂) and prostaglandin (PGI₂) during liver transplantation in end-stage liver disease patients.

METHODS: Twenty-seven patients with end-stage cirrhosis undergoing liver transplantation were enrolled in this prospective study. Blood samples were obtained from superior vena at five different surgical stages. Plasma concentrations of nitrate and nitrite were determined to reflect plasma NO levels. Plasma levels of ET-1, 6-keto-PGF₁ alpha and thromboxane B₂ (TXB₂), the latter two being stable metabolites of PGI₂ and TXA₂ respectively, were measured.

RESULTS: The NO level decreased significantly after vascular cross-clamping and increased significantly at 30 min after reperfusion. While the ET levels at 30 min after clamping and after reperfusion were significantly elevated. The ratio of NO/ET decreased significantly at 30 min after vascular cross-clamping and at the end of surgery. The PGI₂ level and the TXA₂ during liver transplantation were significantly higher than the baseline level, but the ratio of TXA₂/PGI₂ decreased significantly at 30 min after clamping.

CONCLUSION: NO/ET and TXA₂/PGI₂ change during liver transplantation. Although the precise mechanism remains unknown, they may play a role in the pathobiology of a variety of liver transplant-relevant processes.

INTRODUCTION

Liver transplantation is becoming a more and more common treatment method of end-stage liver disease. The hemodynamic alterations occurring after graft reperfusion and the subsequent complications may result from the changes of some biotic activators^[1]. Nitric oxide (NO) and endothelin (ET), thromboxane A₂ (TXA₂) and prostaglandin (PGI₂) are two groups of important vasoactive substance. The clinical and animal experiment documented that the two groups of substance are involved in a variety of liver transplant-relevant processes, including ischemia-reperfusion injury, acute cellular rejection, and circulatory changes characteristic of advanced liver disease^[2]. However, there is considerable controversy in the perioperative plasma concentrations of the two groups of substance and the precise mechanism of these changes is not fully known.

This study monitored blood levels of NO and ET, TXA₂ and PGI₂ at different time points perioperatively in cirrhotic patients undergoing liver transplantation.

MATERIALS AND METHODS

Patients

During a 10-mo period, all patients with end-stage cirrhosis undergoing liver transplantation were enrolled in this prospective study. With the approval of the institutional ethics committee from Sun Yet-Sen University, informed consent was obtained from patient before each assessment. We excluded patients receiving liver transplantation more than one time and patients losing blood more than 3500 mL during operation.

Anesthesia was induced with midazolam (0.05-0.1 mg/kg), propofol (1-2 mg/kg) and fentanyl (5 μ g/kg) intravenously (IV) and neuromuscular blockade was accomplished with nocurium (0.1 mg/kg). Mechanical ventilation was performed with O₂, using a tidal volume necessary to maintain ETCO₂ tension between 4-4.66 kPa(30-35 mmHg). Anesthesia was maintained with both isoflurane at a concentration of 1.0%-3.5% (inhalation) and intermittent fentanyl (50-100 μ g, IV). After induction of anesthesia, a continuous dopamine infusion (2-6 μ g/kg per min) was administered. Blood samples were obtained from the superior vena cava at five different surgical stages: induction of anesthesia (T1), 60 min after beginning of operation (T2), 30 min after vascular cross-clamping (T3), 30 min postreperfusion of new liver (T4) and at the end of surgery (T5). Plasma concentrations of nitrate and nitrite, two metabolites of NO, were determined to reflect plasma NO levels. Plasma ET-1 levels were measured by radio-immunoassay (RIA) and systemic 6-keto-PGF₁ alpha and TXB₂, stable metabolites of PGI₂ and TXA₂ respectively, were measured by RIA also.

Detection of NO

Two milliliters of superior vena cava blood were collected and placed in ice-cooled polypropylene tubes. These samples were immediately centrifuged at 3000 r/min for 10 min and stored at -40°C for late assay. Test kits (Science and Technology Development Center of The General Hospital of PLA, Beijing, China) for NO analysis were used. The optical density value was read in a spectrophotometer set at 546 nm. The NO numeric value was calculated based on a standard and empty tube according to the equation. The calculated results were expressed as μ mol/L.

Detection of ET

Two milliliters of superior vena cava blood were collected and placed in ice-cooled polypropylene tubes containing 40 μ L aprotinin and 30 μ L EDTA. These samples were immediately centrifuged at 3000 r/min for 10 min. Collected plasma was stored at -40°C until batched assays were performed. Plasma ET levels were evaluated with ET detection kit (Science and Technology Development Center of The General Hospital of PLA, Beijing, China) according to the manufacturer's instructions.

Detection of TXA₂ and PGI₂

Three milliliters of superior vena cava blood were collected with a five-milliliter syringe containing 200 μ L EDTA 2Na, mixed fully and injected into a tube. These tubes were immediately centrifuged at 3500 r/min for 15 min. Collected plasma was stored at -40°C until use. Detection of the level of TXA₂ and PGI₂ was performed according to the manufacturer's instructions (Science and Technology Development Center of The General Hospital of PLA, Beijing, China).

Statistical analysis

Data are shown as mean \pm SD. Data of the various time points were compared using ANOVA analysis (SPSS statistical package, version 11.0). Significant difference was accepted at $P < 0.05$.

Table 1 Changes of NO₂/NO₃ and ET levels in plasma during liver transplantation (mean \pm SD, $n = 27$)

	NO ₂ /NO ₃ (μ mol/L)	ET (ng/L)	NO/ET
T1	27.45 \pm 5.70	61.21 \pm 25.38	0.54 \pm 0.25
T2	35.19 \pm 7.72 ^a	74.84 \pm 29.22	0.49 \pm 0.25
T3	25.64 \pm 5.64 ^c	110.61 \pm 24.65 ^{a,c}	0.24 \pm 0.07 ^{a,c}
T4	35.30 \pm 11.23 ^{a,c}	114.00 \pm 26.73 ^{a,c}	0.32 \pm 0.13 ^a
T5	33.86 \pm 8.94 ^c	116.92 \pm 27.08 ^{a,c}	0.28 \pm 0.07 ^{a,c}

^a $P < 0.05$ vs T1; ^c $P < 0.05$ vs T2; ^c $P < 0.05$ vs T3. T1: induction of anesthesia; T2: 60 min after operation beginning; T3: 30 min after vascular cross-clamping; T4: 30 min postreperfusion of new liver; T5: the end of operation.

Table 2 Changes of TXB₂ and 6-keto-PGF₁ levels in plasma during liver transplantation (mean \pm SD, $n = 27$)

	6-keto-PGF ₁ (ng/L)	TXB ₂ (ng/L)	TXB ₂ /6-keto-PGF ₁
T1	152.94 \pm 68.67	118.27 \pm 63.99	0.89 \pm 0.37
T2	932.71 \pm 25.66 ^a	297.45 \pm 127.13 ^a	0.48 \pm 0.25
T3	895.83 \pm 300.71 ^a	265.15 \pm 127.34 ^a	0.33 \pm 0.14 ^a
T4	620.94 \pm 282.41 ^{a,c,c}	271.26 \pm 140.48 ^a	0.49 \pm 0.21 ^c
T5	591.12 \pm 336.57 ^{a,c,c}	208.32 \pm 90.98 ^{a,c,c}	0.43 \pm 0.20

^a $P < 0.05$ vs T1; ^c $P < 0.05$ vs T2; ^c $P < 0.05$ vs T3. T1: induction of anesthesia; T2: 60 min after operation beginning; T3: 30 min after vascular cross-clamping; T4: 30 min postreperfusion of new liver; T5: the end of operation.

RESULTS

A total of 27 patients (81% men, age 49 \pm 11 year, BSA 1.72 \pm 0.15) were enrolled in this study. Among these patients, 15 had hepatitis B virus associated cirrhosis, 12 had cirrhosis and liver cancer.

The changes of nitrate, nitrite and ET levels in plasma during liver transplantation are shown in Table 1. The mean baseline value of NO (reflected by nitrate and nitrite) was 27.45 \pm 5.70 μ mol/L. The NO level decreased significantly after vascular cross-clamping, from 35.19 \pm 7.72 μ mol/L to 25.64 \pm 5.64 μ mol/L ($P < 0.05$). At 30 min after reperfusion, a significant elevation of NO was found, from 25.64 \pm 5.64 μ mol/L to 35.30 \pm 11.23 μ mol/L ($P < 0.05$).

The baseline ET concentration was 61.21 \pm 25.38 ng/L. Compared with the baseline level, the ET levels at 30 min after clamping and after reperfusion were significantly elevated (110.61 \pm 24.65 ng/L, 114.00 \pm 26.73 ng/L respectively).

The changes of TXB₂ and 6-keto-PGF₁ levels in plasma during liver transplantation are shown in Table 2. The prostaglandin level and the TXA₂ level presented the same changes. The concentrations at every time point were significantly higher than the baseline level. Peak levels of prostaglandin were 932.71 \pm 25.66 ng/L and 895.83 \pm 300.71 ng/L, detected at 60 min after operation and 30 min after vascular cross-clamping respectively.

The mean baseline value of NO/ET was 0.54 \pm 0.25. The ratio decreased significantly at 30 min after vascular cross-clamping and at the end of surgery. The mean

baseline value of TXA₂/ PGI₂ was 0.89 ± 0.37 , the ratio decreased significantly at 30 min after clamping. Compared with the value at 30 min after clamping, the ratio increased significantly at 30 min after reperfusion, from 0.33 ± 0.14 to 0.49 ± 0.21 .

DISCUSSION

In this study we measured systemic NO and ET, TXA₂ and PGI₂ at five different surgical stages: basal, hepatectomy, anhepatic, 30 min after graft reperfusion and the end of surgery. Overall results showed that the levels of these two groups of vasoactive substances changed at each stage during liver transplantation. Previous studies have demonstrated that NO and ET, TXA₂ and PGI₂ play an important role in the pathobiology of ischemia-reperfusion injury and postreperfusion syndrome^[3,4]. It suggests that disturbed balance between these vasodilators and vasoconstrictors may contribute to some liver transplantation-relevant syndromes.

NO and ET are the most important local vasodilator and vasoconstrictor respectively. They seem to play a role in almost every organ and tissue. However, there is considerable confusion in understanding their roles. Some researches suggest that the important factors in determining the beneficial versus harmful effects of NO are the amount, duration, and site of NO production^[5]. Ovadia *et al* demonstrated that the fetal pulmonary vasoconstriction after acute constriction of the ductus arteriosus is mediated by NO/ET-1 interactions^[6]. Shirakami *et al* reported that the plasma ET level was increased before transplantation compared with that of healthy children, but decreased during the anhepatic phase^[7]. It increased again after reperfusion and remained at high level in the early postoperative period. These suggest that ET production in the cirrhotic liver is augmented and ET plays some role in circulatory regulation during the perioperative period of pediatric liver transplantation. In the present study, although both NO and ET increased after graft reperfusion, the ratio of them was decreased. It suggested that the imbalance of NO and ET level may participate in the pathophysiology of systemic and local circulation disorders.

Many data about the relationship between another group of vasoactive substances, PGI₂/TXA₂ and hemodynamics have been reported^[8,9]. TXA₂ is both a vasoconstrictor and a potent stimulus for platelet aggregation. Its effect is antagonized by prostacyclin, which is released by vascular endothelial cells. Prostacyclin exerts a variety of effects on the cardiovascular system, including a decrease in blood pressure associated with a decrease in systemic vascular resistance. In clinical liver transplantation, Khoury *et al*^[10] demonstrated 60% of patients undergoing orthotopic liver transplantation accumulated prostacyclin in the portal vein, which could be one of the causes of hypotension seen at reperfusion of the donor liver. As previously demonstrated, we found that the baseline levels of PGI₂ and TXA₂ in our patients

were higher than normal values. It was most likely due to the decreased metabolism of them in patients with end-stage liver disease. In addition, we found PGI₂ and TXA₂ had the same changes during liver transplantation. The levels of PGI₂ and TXA₂ after reperfusion were elevated compared with the baseline level. However, the ratio of TXA₂ and PGI₂ was significantly lower than that of pre-reperfusion. This indicated that the disorder of TXA₂ and PGI₂ might also be involved in the circulation disorders during orthotopic liver transplantation.

In summary, two groups of endogenous vasoactive substance, NO/ET and TXA₂/PGI₂, are changed during liver transplantation. Although the precise mechanism remains unknown, they may play a role in the pathobiology of a variety of liver transplant-relevant processes.

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RAPID COMMUNICATION

Mistletoe alkali inhibits peroxidation in rat liver and kidney

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effect against CCl₄ toxicity by inhibiting the oxidative damage and stimulating GST activities. Thus, clinical application of MA should be considered in cases with carbon tetrachloride-induced injury.

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Key words: Mistletoe alkali; Inhibition of peroxidation; Free radical; Liver and kidney

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Abstract

AIM: To explore the antioxidant and free radical scavenger properties of mistletoe alkali (MA).

METHODS: The antioxidant effect of mistletoe alkali on the oxidative stress induced by carbon tetrachloride (CCl₄) in rats was investigated. The rats were divided into four groups ($n = 8$): CCl₄-treated group (1 mL/kg body weight), MA -treated group (90 mg/kg), CCl₄+MA-treated group and normal control group. After 4 wk of treatment, the level of malondialdehyde (MDA), a lipid peroxidation product (LPO) was measured in serum and homogenates of liver and kidney. Also, the level of glutathione (GSH), and activities of glutathione reductase (GR), glutathione peroxidase (GSPx), superoxide dismutase (SOD), and glutathione-S-transferase (GST) in liver and kidney were determined. Scavenging effects on hydroxyl free radicals produced *in vitro* by Fenton reaction were studied by ESR methods using 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) as a spin trap reagent and H₂O₂/UV as the OH· source. Urinary 8-hydroxydeoxyguanosine (8-OHdG) was determined by competitive ELISA.

RESULTS: In CCl₄-treated group, the level of LPO in serum of liver and kidney was significantly increased compared to controls. The levels of GSH and enzyme activities of SOD, GSPx and GR in liver and kidney were significantly decreased in comparison with controls. In CCl₄+MA-treated group, the changes in the levels of LPO in serum of liver and kidney were not statistically significant compared to controls. The levels of SOD, GSPx and GR in liver and kidney were significantly increased in comparison with controls. There was a significant difference in urinary excretion of 8-OHdG between the CCl₄-treated and MA-treated groups.

CONCLUSION: Oxidative stress may be a major mechanism for the toxicity of CCl₄. MA has a protective

INTRODUCTION

Recently, great attention has been focused on the role of the antioxidative defense system in oxidative stress. Endogenous antioxidants in medicinal herbs may play an important role in antioxidative defense against oxidative damage, possibly protecting the biological functions of cells^[1]. There is increasing interest in the protective biological function of natural antioxidants contained in Chinese medicinal herbs, which are candidates for the prevention of oxidative damage^[2,3].

Viscum coloratum (Komar.) Nakai has long been categorized as a traditional Chinese medicine in China, USA and other countries. A number of lipid-soluble antioxidants have been isolated from *Viscum coloratum* (Komar.) Nakai, such as mistletoe alkali, thionins, glucoprotein, polysaccharose^[4,5]. In the present study, we investigated the antioxidant activities of mistletoe alkali using a rat model of carbon tetrachloride (CCl₄)-induced hepatotoxicity and nephrotoxic nephritis, which could assess *in vivo* antioxidant activities of Chinese medicinal herbs.

Lipid peroxidation is one of the reactions induced by oxidative stress and is especially active in tissues rich in polyunsaturated fatty acid. There have been few comparative studies on the antioxidant ability of traditional Chinese medicine to modify the susceptibility of organs or tissues to oxidative stress and to alter the cellular antioxidant defense system^[6,7]. We have recently found that some compositions of *Viscum coloratum* (Komar.) Nakai can act effectively *in vitro* as antioxidants and peroxyl radical scavengers. Mistletoe alkali is one of these antioxidant compositions.

As the liver is the main target organ of CCl₄, and the

kidney is the main site of CCl₄ accumulation, the present study was to investigate the oxidative status of both organs in rats simultaneously exposed to CCl₄. To determine the ability of mistletoe alkali to act as antioxidants *in vivo*, we fed rats with mistletoe alkali (90 mg/kg per day) and examined the metabolism of this compound as well as its effects on oxidative stress *in vitro*.

MATERIALS AND METHODS

Mistletoe alkali preparation

Viscum coloratum (Komar.) Nakai was immersed into the acidity aqueous solution and smashed to particles, and then the alkaloid was taken out by precipitation with alkaline. In brief, *Viscum coloratum* (Komar.) Nakai was smashed to particles and soaked in the acidity aqua for 48 h, the liquid was added to the alkali to precipitate and remove infusible part and the alkali was added again to precipitate its total alkali. The acidity aqua of *Viscum coloratum* (Komar.) Nakai was proceeded to precipitate the different alkalinity with the aqua.

CCl₄-induced hepatotoxicity and nephrotoxic nephritis

Female Sprague Dawley rats (200-250 g) were maintained in a 12-h light/dark cycle at 22°C with free access to food and water. Vitamin E was used as positive control. In each experiment, the animals were randomly assigned into groups ($n = 8$). In the pretreatment groups, the animals were treated intragastrically with the drug preparation at a desired daily dose for 6 d. Mistletoe alkali and vitamin E were dissolved or suspended in olive oil. Twenty-four hours after the last dosing, the animals were administered intragastrically with CCl₄ (11% (v/v) in olive oil at a dose of 1 mL/kg. Control animals were given the appropriate vehicle, and all treatments were conducted between 900 and 1000 h to minimize variations in animal response due to circadian rhythm. The animals were killed 24 h after CCl₄ treatment. After 2 wk of mistletoe alkali treatment, the rats were starved for 12 h and killed by anesthetization with diethyl ether. Blood was taken from the abdominal aorta and plasma was separated. The kidneys, liver, stomach, large and small intestines were removed and washed with cold PBS. Plasma and tissue homogenates were stored at -80°C. Protein concentrations were determined with bicinchonic acid (BCA) protein assay kit (Pierce, Rockford, IL) with bovine serum albumin as the standard.

Chemicals

Vitamin E, 5,5-dimethyl pyrrole-N-oxide (DMPO), thiobarbituric acid (TBA) were bought from the Sigma Company (USA). "8-OHdG Check" kit was purchased from Fukuroi (Japan) for the control of aging. All other chemicals were of analytical grade.

Hydroxyl radical scavenging assay by electron spin resonance trapping method

Hydroxyl radical (\cdot OH) scavenging activity of mistletoe alkali was determined by spin trapping ESR using DMPO as a spin trap reagent as previously described^[8]. Both

Fenton reaction and H₂O₂/UV system were used as the \cdot OH source. Comparative study on the \cdot OH scavenging activity of mistletoe alkali was carried out in the presence of liver and kidney homogenates and serum using the Fenton system as \cdot OH generator. Briefly, rat liver and kidney homogenates (0.923 mg protein) were incubated with an aliquot of mistletoe alkali at 37°C for 30 min. The homogenates (100 mL) were mixed with 160 mL of distilled water, 30 mL of 100 mol/L H₂O₂ and 30 mL of 200 mol/L DMPO. Then, 30 mL of 2 mmol/L FeSO₄ was finally added to the mixture to initiate the Fenton reaction. DMPO-OH adduct formation was measured in a silica flat cell 2 min after the addition of FeSO₄ using a JEOL JESTE 200 electron spin resonance (ESR) spectrometer (X-Band Microwave Unit). The spectrometer settings were as follows: microwave power: 8 mW; microwave frequency: 9.20 GHz; modulation amplitude: 0.1 mT; time constant: 0.03 s; sweep time: 30 s; center fields: 332.6/322.6 mT.

TBARS formation determination

Briefly, an aliquot of liver and kidney homogenates was mixed with 0.2 mL of 8.1% SDS, 1.5 mL of 20% acetic acid and 1.5 mL of 0.8% TBA, then the volume was adjusted to 4.0 mL with distilled water. After boiled at 95°C for 60 min, the reaction solution was extracted with 1.0 mL of distilled water and 5.0 mL of *n*-butanol and pyridine (15:1 v/v). The absorbance at 532 nm of the organic layer was determined after centrifugation. The whole procedure was performed as previously described^[9].

GSH-Px activity determination

Briefly, an aliquot of liver and kidney homogenates (4 mg wet tissue) in 0.05 mol/L phosphate buffer containing 1.15% (w/v) KCl was mixed in a quartz cuvette with 935 mL of the coupling solution prepared by dissolving 33.6 mg disodium EDTA, 6.5 mg Na₂N₃, 30.7 mg of GSH, 16.7 mg NADPH and 100 units of GSH reductase in 100 mL of 50 mmol/L Tris-HCl (pH 7.6). Kinetic decay of NADPH fluorescence (Ex. 355 nm/Em. 465 nm) was measured after the addition of 25 mL of 1 mmol/L H₂O₂ as substrate using a Hitachi model 650-60 fluorescence spectrophotometer. The whole procedure was carried out as previously described^[10].

SOD activity measurement

One unit of SOD activity was defined as the amount of enzyme causing 50% inhibition of epinephrine auto-oxidation to adrenochrome. For Mn SOD activity measurement 1 mmol/L KCN was added to the reaction mixture to inhibit CuZn SOD activity which was approximated by subtracting Mn SOD activity from total SOD activity as previously described^[11]. Protein content was assayed by the Bradford method (Bio-Rad, Hercules, Calif., USA).

Determination of urinary 8-OHdG by competitive ELISA

Urine samples were centrifuged at 10 000 r/min for 15 min, and the supernatant was used for determination of 8-OHdG by competitive ELISA using the "8-OHdG Check" kit. The determination range was 0.64-2000 µg/

Table 1 LPO, GSH-Px, SOD in rat liver ($n = 32$, mean \pm SD)

Groups	Dosage (mg/kg)	LPO (nmol/mg protein)	GSH-Px (nmol/mg protein)	SOD (nmol/mg protein)
Normal control	-	11.142 \pm 0.916 ^b	18.713 \pm 3.587 ^b	31.537 \pm 2.848 ^b
CCl ₄ + MA group	90	10.326 \pm 1.213 ^b	20.403 \pm 2.388 ^c	39.901 \pm 2.667 ^c
CCl ₄ + vit E group	400	11.221 \pm 2.182 ^b	16.353 \pm 2.962 ^b	27.736 \pm 3.646 ^b
CCl ₄ group	-	17.271 \pm 1.108	13.342 \pm 3.636	21.639 \pm 3.991

^b $P < 0.01$, ^c $P < 0.05$ vs CCl₄ group.

Table 2 LPO, GSH-Px, SOD in rat kidney ($n = 32$, mean \pm SD)

Groups	dosage (mg/kg)	LPO (nmol/mg protein)	GSH-Px (μ /g mL)	SOD (μ /mg protein)
Normal control	-	1.268 \pm 0.99 ^b	7.174 \pm 0.576 ^c	39.573 \pm 7.322 ^c
CCl ₄ + MA group	90	1.973 \pm 0.512 ^b	7.431 \pm 0.483 ^c	37.887 \pm 6.979 ^b
CCl ₄ + Vit E group	400	1.312 \pm 0.418 ^b	6.698 \pm 0.746 ^b	39.399 \pm 8.684 ^c
CCl ₄ group	-	3.826 \pm 0.289	4.576 \pm 0.611	22.639 \pm 5.342

^b $P < 0.01$, ^c $P < 0.05$ vs CCl₄ group.

Table 3 MDA, SOD, GSH-Px in blood plasma ($n = 32$, mean \pm SD)

Groups	Dosage (g/kg)	MDA (mol/L)	GSH-Px (μ /mL)	SOD (μ /mL)
Norma control	-	14.341 \pm 2.01	21.17 \pm 3.82 ^b	19.01 \pm 2.36 ^b
CCl ₄ + MA group	90	12.88 \pm 3.98 ^b	22.67 \pm 3.42 ^b	23.62 \pm 3.17 ^c
CCl ₄ + Vit E group	400	14.91 \pm 2.79	18.77 \pm 1.98 ^b	17.63 \pm 1.88 ^b
CCl ₄ group	-	14.98 \pm 3.63	16.11 \pm 2.41	14.88 \pm 3.15

^b $P < 0.01$, ^c $P < 0.05$ vs CCl₄ group.

Table 4 Effects of mistletoe alkali on Fenton and H₂O₂/UV-mediated DMPO-OH formation

Drug concentration (μ g/mL)	Sample size (n)	Opposite high peak of ESR (mean \pm SD)	Clearance rate (%)
0	20	38.71 \pm 3.62	-
2	20	34.16 \pm 3.75	11.67
10	20	29.42 \pm 2.99	47.82
50	20	22.65 \pm 4.83	67.67
100	20	14.38 \pm 2.76	91.28

mL and the specificity of the monoclonal antibody used in the competitive ELISA kit was established.

Statistical analysis

Significance was tested by the paired *t*-test or analysis of variance. Results were presented as mean \pm SD. $P < 0.05$ was considered statistically significant.

RESULTS

MDA and LPO formation in liver and kidney homogenate and plasma was considerably increased in the model group. However, in the mistletoe alkali-treated groups, no significant increase in MDA and LPO formation was observed (Table 1, Table 2 and Table 3). Results showed that hepatotoxicity and nephrotoxic nephritis in rats were induced by CCl₄ with formation of lipid peroxide. However, mistletoe alkali was effective in reducing malonyl dialdehyde (MDA) content in the tissues, and had good scavenging effects on hydroxyl free radicals.

The effective protection of liver and kidney against oxidative damage induced by mistletoe alkali was further demonstrated by determining GPx and SOD activity in the Liver and kidney homogenate and plasma. GPx and SOD activity decreased markedly in the liver and kidney homogenate and plasma in model group, but activity loss was prevented by mistletoe alkali in the pretreatment groups. GPx and SOD activity increased slightly compared to normal group. The data in the present study suggested that mistletoe alkali could be a potential herbal medicine for improving GPx and SOD activity in liver and kidney tissue and plasma. The enzymes level is shown in Table 1, Table 2 and Table 3. The scavenging activity was steadily increased with the increase of drug density, suggesting that the free radical scavenging activity was related with

mistletoe alkali density.

The DMPO-OH signal produced by the Fenton reaction was reduced by the addition of mistletoe alkali in a concentration dependent manner (Table 4). The 50% inhibitory concentration of mistletoe alkali for DMPO-OH formation was compared to that for Trolox as a reference radical scavenger. The results indicated that mistletoe alkali not only scavenged \cdot OH directly but also inhibited \cdot OH generation in the Fenton reaction. This was further confirmed when the \cdot OH scavenging activity of mistletoe alkali was determined in the H₂O₂/UV system as \cdot OH source. In this system, the Trolox equivalence of the mistletoe alkali solution was about 0.1 mmol/L (data not shown).

There was a significant difference in urinary excretion of 8-OHdG between the control group and 900 mg/kg mistletoe alkali-fed group (7.84 \pm 3.72, 3.26 \pm 1.92 μ mol/d, $P < 0.05$).

DISCUSSION

There is a growing body of evidence that oxygen-derived free radicals are involved in the pathogenesis of over 50 human diseases^[12]. Antioxidant therapy aimed at reducing free radical-mediated tissue damage represents a rational approach in preventing the onset and/or progression of free radical-related tissue damage. In this connection, the measurement of antioxidant activity should form an additional basis for drug screening and selection^[13]. In the present study, we examined the antioxidant potential of a traditional Chinese medicine, *Viscum coloratum* (Komar.) Nakai.

Recently, rapid progress has been made in research of *Viscum coloratum* (Komar.) Nakai. The preparation of *Oujisheng* has already been applied extensively in

treatment of cancer^[14]. China has a long history in research of *Viscum coloratum* (Komar.) Nakai. In 1934, Jingli Tong discovered that the Chinese *Viscum coloratum* (Komar.) Nakai (Huangguo *Viscum coloratum* (Komar.) Nakai) can lower blood pressure^[15].

The involvement of free radical-mediated oxidative process in the development of CCl₄ hepatotoxicity is well established^[16]. It was reported that impairment of hepatic antioxidant status is associated with a substantial hepatocellular damage induced by CCl₄^[17]. GSH is a crucial determinant of tissue susceptibility to oxidative damage, and the depletion of hepatic GSH content has been shown to be associated with an enhanced toxicity to chemicals including CCl₄^[18]. The liver protection afforded by pretreatment with NAC, a precursor for GSH synthesis, and vitamin E, an inhibitor of lipid peroxidation, indicates that both the sustained hepatic GSH content and the lipid peroxidation inhibition are important factors involved in protecting against CCl₄ hepatotoxicity. Changes in the sensitivity of tissue homogenates to *in vitro* oxidative challenge have been used as an indicator of altered tissue susceptibility to free radical-induced oxidative injury *in vivo*^[19]. Our results, indicating an increase in sensitivity of tissue homogenates to ferric ion-induced lipid peroxidation following CCl₄ treatment, have validated the application of "forced peroxidation" assays in measuring alterations in tissue after oxidative challenge.

To our knowledge, there is no report indicating that the use of MA *in vivo* studies counteracts CCl₄ toxicity. Mistletoe alkali pretreatment at a daily dose of 900 mg/kg body weight for 6 d significantly enhanced hepatic antioxidant status in CCl₄-treated rats, with a concomitant reduction of hepatocellular damage, suggesting that the involvement of GSH-enhancing action can protect liver against damage.

8-OHdG, a DNA base-modified product generated by reactive oxygen species, is mutation prone and a good marker for oxidative damage. It has been hypothesized that oxidative damage can occur in DNA during the peroxidative breakdown of membrane polyunsaturated fatty acids. Lipid peroxidation can mediate 8-OHdG formation *in vitro* and may play a role in carcinogenesis by inducing 8-OHdG generation *in vitro*. In this study, the amount of 8-OHdG in the urine of rats fed with mistletoe alkali was significantly lower than that in the control rats, suggesting that mistletoe alkali plays a role in the prevention of oxidative DNA damage *in vivo*.

In conclusion, increased level of free radicals is associated with decreased antioxidant status in liver and kidney of rats treated with CCl₄. This finding is similar with other investigations^[20,21]. Mistletoe alkali strongly inhibits lipid peroxidation and thereby decreases liver and kidney damage.

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RAPID COMMUNICATION

Expression of T-STAR gene is associated with regulation of telomerase activity in human colon cancer cell line HCT-116

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Abstract

AIM: To investigate the effects on telomerase activity of transfection of human T-STAR gene full-length sense cDNA or partial antisense cDNA into human colon cancer cell line HCT-116.

METHODS: mRNA and protein expression levels of T-STAR gene were determined by RT-PCR and western blot, and telomerase activity was measured by PCR-ELISA, after transfection of T-STAR sense or antisense gene into HCT-116 cells with lipofectamine.

RESULTS: T-STAR gene expression was enhanced or knocked down both at mRNA and protein levels, and telomerase activity was significantly increased or decreased.

CONCLUSION: The T-STAR gene may participate in regulation of telomerase activity in human colon cancer HCT-116 cells in a parallel fashion.

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Key words: T-STAR; Telomerase; Human colon cancer cells; Cell transfection

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INTRODUCTION

T-STAR, a recently cloned member of STAR gene family, is highly expressed in testis, muscle, and brain and contains a STAR domain, an RNA-binding domain present in a growing family of proteins involved in developmental processes^[1-3]. The protein can bind to a variety of signal-transducing proteins and may act as an adaptor in signal-transduction pathways. T-STAR was identified as a protein interacting with the protein RNA-binding motif (RBM) in spermatogenesis. Down-regulation of T-STAR expression was found in SV40-transformed immortal fibroblasts, and this protein may interact with telomerase^[4]. In most progressive phase malignant tumor tissues and cancer cell lines, the telomerase activity maintained at a high level, whereas, at a lower or undetectable level in normal cells. The activation of telomerase is involved in tumorigenesis and progression^[5-8]. In this study, we transfected the sense or antisense T-STAR gene into human colon cancer cell line HCT-116 to overexpress or inhibit T-STAR protein, and to further observe whether T-STAR gene participates in the regulation of telomerase activity in HCT-116 cells.

MATERIALS AND METHODS

Materials

The eukaryotic expression plasmid, pcDNA3.1 was purchased from Invitrogen. Human T-STAR full length cDNA was a gift by Dr. Chew of Leicester University. The full length cDNA of T-STAR was inserted into pcDNA3.1 vector at *EcoRI* site in a sense orientation to generate a recombinant sense expression plasmid, pcDNA-STAR. The fragment of T-STAR cDNA digested by *ApaI* restriction enzyme was ligated in the *ApaI* site of pcDNA3.1 in an antisense orientation to construct a recombinant antisense plasmid, pcDNA-asSTAR. Human colon cancer cell line HCT-116 was provided by Department of Abdominal Surgery of Southwest Hospital, Chongqing. The transfection reagent of Lipofectamine2000 was purchased from Invitrogen. RPMI1640, fetal calf serum, and other reagents for cell culture were purchased from Hyclone. Monoclonal antibody against T-STAR, mAbT was purchased from Santa Cruz Biotech. Rabbit antigoat IgG polyclonal antibodies HRP-conjugated were purchased from Beijing Zhongshan Biotech (China). RNA PCR reagent kit was

from Dalianbo Biotech. Telomerase PCR ELISA reagent kit was from Roche.

Methods

Human colon cancer cell line HCT-116 cells were maintained in RPMI1640 medium, supplemented with 10% heat-inactivated fetal calf serum, 100 U/mL of penicillin and 100 µg/mL of streptomycin and were grown at 37°C, in a humidified incubator containing 5 mL/L CO₂. Transfection was performed according to the LipofectAMINE2000 method (Invitrogen) using 2 µg of each plasmid DNA (empty vector pcDNA3.1 as control, antisense plasmid pcDNA-asSTAR and sense plasmid pcDNA-STAR as experimental group). Briefly, adherent cells were seeded in six-well plates (5 × 10⁵ cells per well) grown overnight to 50%-60% confluency and were washed three times with phosphate-buffered saline, and medium was replaced with 500 µL of OPTI-MEM medium before a mixture of LipofectAMINE and DNA was added to the well for 8 h. And then the medium containing transfection mixture was replaced with 2 mL fresh RPMI1640. After 24 h of transfection, the cells were selected in RPMI1640 supplemented with 500 µg/mL G418 (Geneticin, GIBCO) for 10 d. Furthermore, the cells of selected single colonies were grown in same medium with 200 µg/mL G418 for another two weeks. Total RNA was extracted from each group of transfected cells using TRIzol reagent (Invitrogen Life Technologies). Concentration and purity were confirmed by spectrophotometry and electrophoresis on the denaturing formaldehyde-agarose gel. Reverse transcription reactions were performed on 5 µg of the isolated total RNA using the ProSTAR RT-PCR kit (Stratagene, La Jolla, CA). The following specific primers were used for PCR assay, for human T-STAR gene: P1-5'CAGGATGGGACATGCTTTG3', P2-5'TCTGTAGACGCCCTTTGCT3', for inner control β-actin: P1-5'GTGGGC CGCTCTAGGCACCAA3', P2-5'CTCTTTGATGTCACGCACGATTTTC3'. All PCR reactions were incubated at 95°C for 5 min, and then cycled at 95°C for 45 s, 48°C for 30 s and 72°C for 1 min for 35 cycles and, finally, at 72°C for 10 min for extension. The PCR products were electrophoresed on 10 g/L agarose gel to calculate the amount of T-STAR mRNA expression. The densities of target DNA bands of each group on the electrophoresis were analysed by software of Quantity One, and was normalized to inner control β-actin. The relative quantities are given as mean ± SD. The experiment was done in triplicates. Total cellular proteins were isolated from each group of transfected cells. Briefly, 90% confluency live cells were washed twice with 10 mmol/L PBS, and then were completely lysed with 200 µL 1 × SDS loading buffer. The lysate was transferred to a new 1.5 mL eppendorf tube, and heated to 100°C for 10 min. Then cellular genomic DNA was broken by sonication. About 10 µL protease inhibitor of PMSF per mL extract was mixed together. Protein concentration was determined according to Bradford, and samples were stored at -20°C. For western blot analysis, 40 µg total cellular proteins were separated on a 4%-15% sodium dodecyl sulfate-polyacrylamide gel. After electrotransfer to nitrocellulose membranes (BioRad),

uniformity of loading and transfer was confirmed by Poncea staining. The membranes were blocked overnight in TS complete (20 mol/L Tris-HCl, 150 mol/L NaCl, 5% non-fat dry milk, and 0.1% Tween 20) at 4°C. Blots were incubated for 1 h at room temperature with T-STAR mAb (Santa Cruz Biotech.) antibodies. Blots were washed and then incubated with horseradish peroxidase-conjugated secondary antibody (1:2000) (Sigma, St. Louis, MO) at room temperature for 2 h. The immune complexes were detected by the automated image documentation systems and bands density was analyzed by Quantity One software. The telomerase activity was measured with PCR ELISA kit^[9].

Statistical analysis

Data are expressed as mean ± SD. Differences between experimental groups were assessed for significance by using the two-tailed unpaired Student's *t* test. A *P* value of < 0.05 was considered to be statistically significant.

RESULTS

Cellular T-STAR mRNA expression level

Three clear bands were present at 5S, 18S and 28S on the electrophoresis of total RNA of each group of cells. The brightness of the band at 28S was about 2-fold compared with that at 18S. There was no significant degradation with the mRNA, which could be applied in further experiments (data not shown). Both cDNA of T-STAR gene and β-actin gene simultaneously being reverse transcribed from the mRNA were electrophoresed on an agarose gel (Figure 1A). The semi-quantitative analysis was applied to the bands of T-STAR cDNA (Figure 1B). Compared with the average expression level of HCT-pcDNA transfected control cell group, T-STAR mRNA expression was increased to 296% in HCT-STAR group (*P* < 0.01), decreased to 59% in HCT-asSTAR group (*P* < 0.01), and remained slightly changed in HCT-116 group (*P* < 0.01).

T-STAR protein expression level

The T-STAR protein could be specifically detected at the molecular weight *Mr*55 on the immuno-blots in all the HCT-116 cell groups transfected with different constructs (Figure 2A). The T-STAR protein amount was enhanced in HCT-STAR group, reduced in HCT-asSTAR group, and not significantly changed in HCT-116 group in contrast to HCT-pcDNA group. Densitometry analysis showed that the T-STAR protein expression was increased about 180.8% in HCT-STAR cells (*P* < 0.01), decreased about 83.80% in HCT-asSTAR cells (*P* < 0.01) compared with HCT-pcDNA cells (Figure 2B).

Telomerase activity

No significant changes of cellular telomerase activity were observed between HCT-116 cell group and HCT-pcDNA transfected cell group (Figure 3). Interestingly, the telomerase activities were significantly decreased in HCT-asSTAR group (*P* < 0.01), in which the expression of T-STAR gene was inhibited; whereas, the telomerase activities were significantly increased in the HCT-STAR group (*P* < 0.05) with up-regulation of T-STAR gene expression.

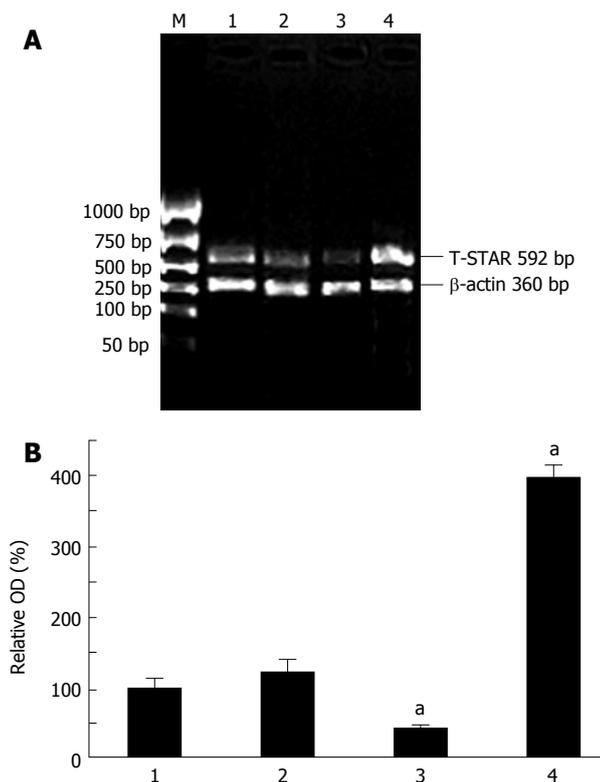


Figure 1 A: The quantitation of T-STAR gene cDNA by RT-PCR from mRNA on electrophoresis of agarose gel; B: The relative expression levels of T-STAR mRNA of each group on electrophoresis of agarose gel, ^a*P* < 0.01 vs 2. M: Marker; 1: HCT; 2: HCT-pcDNA; 3: HCT-asSTAR; 4: HCT-STAR.

DISCUSSION

T-STAR is a 55 kDa protein, one of three members of the SAM68 family of RNA-binding proteins with proline and tyrosine-rich regions that have been shown to be involved in various gene expression pathways including the developmental processes and/or extracellular signals to control the cellular fate of mRNA^[10,11]. T-STAR gene is localized to chromosome 8q24.3, and the full length cDNA ranged in size from 1.2 kb to 1.9 kb. T-STAR is highly expressed in testis, muscle, and brain^[10]. The STAR proteins have multiple functions in pre-mRNA splicing, signalling and cell cycle control. T-STAR generally acts as a growth suppressor, which is down-regulated upon immortalization of many cell lines^[9-11]. Our previous study found that T-STAR may interact with hTERT, participating in processes of cell growth and proliferation. In this study, we successfully established stable colon cancer cell lines in which T-STAR gene was specially up-regulated or down-regulated both at protein and mRNA levels in HCT-STAR cells or HCT-asSTAR cells. Meanwhile, the changes of telomerase activities had a parallel fashion with the expression level of T-STAR in those two cell lines, which was enhanced in HCT-STAR and reduced in HCT-asSTAR. Similar results have been found in our previous study in other tumor tissues or cell lines (unpublished data). The current data further demonstrated that the expression of T-STAR is positively correlated with hTERT and telomerase activity. In other words, it is possible that up-regulation of T-STAR may lead to hTERT and

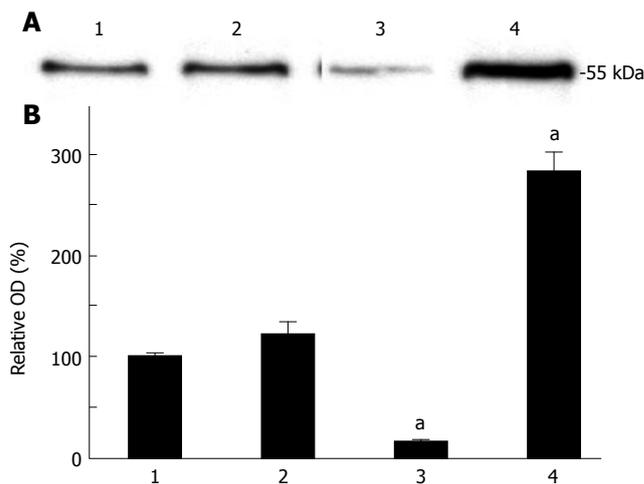


Figure 2 A: Western blot analysis of the T-STAR protein expression level of each group of cells; B: Densitometry analysis of the amount of T-STAR protein expression in each group of cells, ^a*P* < 0.01 vs 2. 1: HCT; 2: HCT-pcDNA; 3: HCT-asSTAR; 4: HCT-STAR.

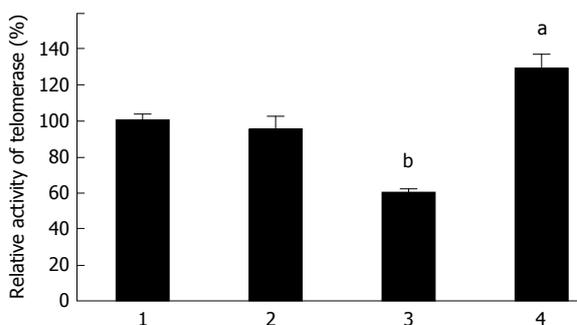


Figure 3 Quantitation analysis of telomerase activity of each HCT cell groups, ^a*P* < 0.05 vs 2, ^b*P* < 0.01 vs 2. 1: HCT; 2: HCT-pcDNA; 3: HCT-asSTAR; 4: HCT-STAR.

telomerase activity increase in tumor cells, and vice versa.

Telomeres are thought to play a critical role in cellular immortalization and carcinogenesis^[12]. Our study on different pathologic types of tumor tissues found that T-STAR mainly expressed in adenoma cells. T-STAR may also be involved in cellular immortalization which is one of the basic characteristics of tumor cells^[2,4,13]. Kool *et al*^[4] have found that T-STAR is down-regulated in SV40-transformed immortalized fibroblasts (VH10/SV) compared with the corresponding nonimmortal SV40-transformed cells. In normal cells, T-STAR expression is clearly present and comparable with expression levels in SV40-transformed precrisis cells. No changes are observed in T-STAR expression as cells progress toward senescence. The down-regulation in the immortalized cells was not restricted to VH10/SV, but was also observed in other fibroblast cell lines transformed by SV40 large T^[15,14]. Interestingly, the common feature of the SV40-transformed cell lines showing down-regulation of T-STAR is that they displayed a distinct proliferative crisis before immortal clones arose. In contrast, both immortal cell lines that do not show abrogation of T-STAR expression, arose without a clear crisis. These data suggest

that loss of T-STAR expression might be a prerequisite to escape from crisis. If immortalization occurs in earlier stages after SV40 transformation, these immortal cells will gradually overgrow the population before crisis and apparently do not need the downregulation of T-STAR. This could indicate that T-STAR plays a role during crisis, and that immortalization cannot occur unless T-STAR expression is down-regulated. T-STAR is likely one of important components in proliferative crisis or immortalization pathway^[15,16]. So far, there is rarely experimental data to investigate the T-STAR expression in cell lines characterized with high telomerase activity and that immortalization has already been completed. Recent observation has shown that the mature male germ cells, as one kind of immortalized cells, with high telomerase activities also maintained abounding T-STAR protein^[2], which is consistent with our results. That is T-STAR expression might be positively correlated with telomerase activity in some kinds of immortalized cells, and T-STAR might be one component in the systems of telomerase activity regulation. Although the exact mechanism of this is unclear yet, from the analysis of T-STAR known functions, it can be speculated that T-STAR might play a role in phosphorylation and pre-mRNA splicing of hTERT.

Telomerase consists of an RNA component, telomerase RNA (TER), which includes the template for synthesis of telomere DNA, and a protein catalytic component, the telomerase reverse transcriptase (TERT), which mediates RNA template-dependent synthesis of telomere DNA. The phosphorylation^[17,18] by PKC, AKT and PTK or dephosphorylation by PP2A for hTERT, the key component of the telomerase complex, can modulate telomerase activity^[19-21]. The interaction of hTERT with c-Ab1 tyrosine kinase binding domain SH3 may result in phosphorylation and inhibit telomerase activity *in vitro* or *in vivo*^[21-23]. Tyrosine phosphorylation of Sam68, the homologues of T-STAR and as an adaptor protein in signal transduction, promotes its interaction with SH2 containing proteins. The association of Sam68 with SH3 domain-containing proteins, and its tyrosine phosphorylation may negatively regulate its RNA binding activity^[24,25]. T-STAR can form hetero-multimers^[4,9,13] with Sam68 to inhibit the interaction of Sam68 with its target protein. So T-STAR might interfere with Sam68 binding with Ab1 SH3 by reverse phosphorylation, and lead to impairment of Ab1 SH3 function of increasing telomerase activity. Another possible mechanism is that T-STAR may take part in the splicing of hTERT pre-mRNA. Different length of hTERT transcripts by alternative-splicing sites exists in cells^[26-28], but only the full length transcripts for hTERT is functional, which is associated with telomerase activity. The selective-splicing for hTERT may be one of the modes that regulate telomerase activity. The 3'-UTR of T-STAR is the binding site for target mRNA^[29]. T-STAR may play a role in processing of target pre-mRNA and selection of splicing sites^[30], and also interact with the protein involved in the selective splicing^[30,31]. We assume that T-STAR might control pre-mRNA splicing for hTERT. The enhancement of full length transcripts for hTERT may up-regulate telomerase activity. However, the exact mechanism needs to be further investigated.

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Dynamic changes of HBV DNA in serum and peripheral blood mononuclear cells of chronic hepatitis patients after lamivudine treatment

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CONCLUSION: Lamivudine has remarkable inhibitory effects on HBV replication both in serum and in PBMCs. The inhibitory effect on HBV DNA in PBMCs is weaker than that in serum.

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Key words: Lamivudine; Hepatitis B virus; DNA; Peripheral blood mononuclear cells

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Abstract

AIM: To study the dynamic changes of hepatitis B virus (HBV) DNA in serum and peripheral blood mononuclear cells (PBMCs) of patients after lamivudine therapy.

METHODS: A total of 72 patients with chronic HBV infection were included in this study. All patients were confirmed to have the following conditions: above 16 years of age, elevated serum alanine aminotransferase (ALT), positive hepatitis B e antigen (HBeAg), positive HBV DNA in serum and PBMCs, negative antibodies against HAV, HCV, HDV, HEV. Other possible causes of chronic liver damages, such as drugs, alcohol and autoimmune diseases were excluded. Seventy-two cases were randomly divided into lamivudine treatment group ($n = 42$) and control group ($n = 30$). HBV DNA was detected both in serum and in PBMCs by fluorescence quantitative polymerase chain reaction (PCR), during and after lamivudine treatment.

RESULTS: In the treatment group, HBV DNA became negative both in serum and in PBMC, of 38 and 25 out of 42 cases respectively during the 48 wk of lamivudine treatment, the negative rate was 90.5% and 59.5% respectively. In the control group, the negative rate was 23.3% and 16.7% respectively. It was statistically significant at 12, 24 and 48 wk as compared with the control group ($P < 0.005$). The average conversion period of HBV DNA was 6 wk (2-8 wk) in serum and 16 wk (8-24 wk) in PBMC.

INTRODUCTION

Lamivudine (LAM) is a new antiviral agent against hepatitis B virus (HBV) infection. It has been shown that lamivudine therapy can rapidly reduce HBV DNA levels in serum and improve liver histology^[1-3]. However, the dynamic changes of HBV DNAs in serum and peripheral mononuclear cells (PBMCs) in patients after lamivudine therapy are not clear, especially in PBMCs.

PBMCs contain various kinds of active immune cells. The activities of immune cells affect directly the results and efficacy of antiviral therapy^[4,5]. Few studies are available on the dynamic changes of HBV DNA in serum and PBMCs. The aim of this study was to investigate the inhibition of HBV DNA in serum and PBMCs in patients after LAM therapy.

MATERIALS AND METHODS

Patients and materials

A total of seventy-two patients with chronic hepatitis B were from Out-patient and In-patient Departments of our hospital during February 2003-February 2004. There were 50 males and 22 females aged 18-60 years (average 32.4 years). Diagnosis of hepatitis B was made according to the revised standard of the diagnosis established at the Tenth National Symposium on Viral Hepatitis in Xi'an in

Table 1 Change of HBV DNA in serum and PBMC of chronic hepatitis B patients after lamivudine treatment *n* (%)

Group	HBVDNA in serum				HBVDNA in PBMC			
	4 wk	12 wk	24 wk	48 wk	4 wk	12 wk	24 wk	48 wk
Lamivudine Treatment 42	9 (21.1)	26 (61.9) ^a	34 (80.9) ^a	38 (90.5) ^a	4 (9.5)	15 (35.7) ^a	20 (47.6) ^a	25 (59.5) ^a
Routine Treatment 30	2 (6.7)	3 (10.0)	5 (16.7)	7 (23.3)	1 (3.3)	2 (6.7)	4 (13.3)	5 (16.7)

^a*P* < 0.005 vs control group.

2000^[6]. All patients were confirmed to have the following conditions: above 16 years of age, normal or abnormal ALT, positive HbsAg and HBeAg in serum, negative anti-HCV, anti-HDV and anti-HEV in serum, positive HBV DNA in serum and PBMCs.

Methods

Seventy-two patients with chronic hepatitis B were randomly divided into two groups: lamivudine treatment group (*n* = 42) receiving 100 mg lamivudine daily for 48 wk, control group (*n* = 30) receiving routine medication with vitamin C and inosine, *etc.* All samples were prepared and stored at -20°C for further examination. HBV DNA in serum and PBMCs was detected after 4, 12, 24 and 48 wk of treatment.

Quantitative determination of HBA DNA in serum and PBMCs

HBV DNA in serum was detected by fluorescence quantitative PCR assay, strictly according to the manufacturer's instructions (Da An Gene Institute, Shenzhen, No: 20030041).

Lymphocyte separation medium (Second Reagent Factory of Shanghai, No. 200304) was used to separate PBMCs. After isolation, the cells were washed three times with PBS containing 1% brine. The cells were diluted to 1×10^6 /mL with RPMI before detection.

Cellular DNA was extracted according to NaI method. Briefly, 100 μ L 1×10^6 /mL cell suspension and 200 μ L NaI were well mixed, inverted for 20 s, then mixed with 400 μ L chloroform/iso-amy alcohol (24:1) and spun by centrifugation at 10 000 r/min for 12 min. Three hundred microlitre supernatant was mixed with 200 μ L pure dimethyl carbinol, spun by centrifugation for 12 min at 14 000 r/min. Supernatant was collected and mixed with 1 mL 70% ethanol, the DNA was precipitated at -20°C.

HBV DNA in PBMCs was also detected by fluorescence quantitative PCR assay. The reaction conditions were: pre-denaturation at 93°C for 3 min, followed by 40 cycles of denaturation at 93°C for 45 s, extension at 55°C for 60 s and a final extension at 93°C for 30 s, at 55°C for 60 s. The reference graph was drawn according to standard content by a computer. The amount of HBV DNA was calculated. During PCR, a strict control was performed. All results were negative.

Statistical analysis

Statistical analysis was carried out by chi square test and *t*-test. *P* < 0.05 was considered statistically significant.

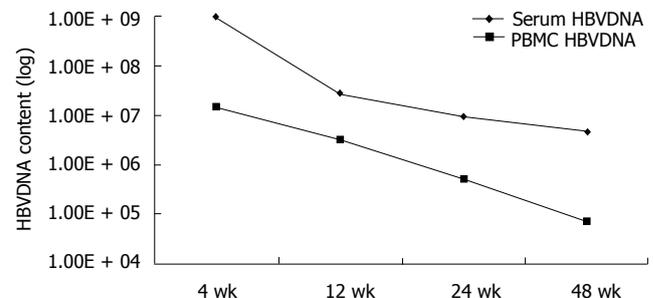


Figure 1 Content of HBV DNA in serum and PBMC of chronic hepatitis B patients after lamivudine treatment.

RESULTS

The content of HBV DNA in serum and PBMC was decreased after lamivudine treatment (Table 1, Figure 1).

DISCUSSION

Lamivudine is a HBV polymerase inhibitor and acts on HBV replication both *in vitro* and *in vivo*. It has been shown that lamivudine can suppress HBV replication, decrease transaminase levels and improve liver histology^[7-10]. However, the main goal of treatment in chronic hepatitis B is to eliminate or persistently inhibit the replication of HBV. After long-term application of lamivudine, antiviral resistance may occur due to HBV polymerase mutation, manifested as increased HBV DNA and serum transaminase, suggesting that the content of HBV DNA can be used to evaluate the efficacy of antiviral therapy. Lamivudine is not able to eliminate HBV ccc DNA in hepatocytes^[11], which is the main reason for HBV DNA rebound after withdrawal. Studies have shown that patients with hepatitis B usually accompany disorder of immune function caused by immunological injury^[12-25]. PBMCs contain various immune active cells, and play a significant role in immune responses during antiviral therapy. It has been shown that HBV infection may exist in PBMCs^[4,26,27]. When PBMCs are infected with HBV, they not only affect the function of immunity, but also weaken the efficacy of antiviral drugs. Therefore, the dynamic change of HBV DNA in PBMCs may be a reliable index for curative antiviral efficacy.

In our study, lamivudine had remarkable inhibitory effects on HBV DNA both in serum and in PBMCs. When the treatment time was prolonged, the negative rate of HBV DNA in serum and PBMCs was gradually increased. The eliminate effect of lamivudine on HBV DNA in

PBMCs was lower than that in serum. The negative rate of HBV DNA in serum was 90.48%, but only 59.52% in PBMCs after lamivudine treatment. HBV DNA was positive in 17 cases of chronic hepatitis B after 48 wk of treatment, which may be associated with the fact that mutations occurred in HBV after LAM therapy^[28], HBV DNA in PBMCs interferes with the immune activity of cells after HBV integrates with PBMCs, and decreases the contents of Ig, C₃, TNF and activity of NK cells as well as the ratio of CD4⁺/CD8⁺^[4,5,29]. Therefore, the function of cell-mediated immunity and humoral immunity are reduced and affect the curative effect of lamivudine.

In conclusion, HBV DNA exists in PBMCs even after 48 wk of lamivudine treatment. The presence of HBV DNA in PBMCs may infect hepatocytes again and cause the relapse of hepatitis.

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RAPID COMMUNICATION

Intestinal permeability of metformin using single-pass intestinal perfusion in rats

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Abstract

AIM: To characterize the intestinal transport and mechanism of metformin in rats and to investigate whether or not metformin is a substrate for P-glycoprotein (P-gp).

METHODS: The effective intestinal permeability of metformin was investigated using single-pass intestinal perfusion (SPIP) technique in male Wistar rats. SPIP was performed in three isolated intestinal segments (duodenum, jejunum and ileum) at the same concentration of metformin (50 $\mu\text{g}/\text{mL}$) to test if the intestinal transport of metformin exhibited site-dependent changes, and in a same isolated intestinal segment (duodenal segment) at three different concentrations of metformin (10, 50, 200 $\mu\text{g}/\text{mL}$) to test if the intestinal transport of metformin exhibited concentration-dependent changes. Besides, P-gp inhibitor verapamil (400 $\mu\text{g}/\text{mL}$) was co-perfused with metformin (50 $\mu\text{g}/\text{mL}$) in the duodenum segment to find out if the intestinal absorption of metformin was affected by P-gp exiting along the gastrointestinal track. Stability studies were conducted to ensure that the loss of metformin could be attributed to intestinal absorption.

RESULTS: The effective permeability values (P_{eff}) of metformin in the jejunum and ileum at 50 $\mu\text{g}/\text{mL}$ were significantly lower than those in the duodenum at the same concentration. Besides, P_{eff} values in the duodenum at high concentration (200 $\mu\text{g}/\text{mL}$) were found to be significantly lower than those at low and medium concentrations (10 and 50 $\mu\text{g}/\text{mL}$). Moreover the co-perfusion with verapamil did not increase the P_{eff} value of metformin at 50 $\mu\text{g}/\text{mL}$ in the duodenum.

CONCLUSION: Metformin could be absorbed from the whole intestine, with the main absorption site at duodenum. This concentration-dependent permeability behavior in the duodenum indicates that metformin is transported by both passive and active carrier-mediated saturable mechanism. The P_{eff} value can not be increased by co-perfusion with verapamil, indicating that absorption of metformin is not efficiently transported by P-gp in the gut wall. Furthermore metformin is neither a substrate nor an inducer of P-gp. Based on the P_{eff} values obtained in the present study and using established relationships, the human fraction dose absorbed for metformin is estimated to be 74%-90% along human intestine.

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Key words: Metformin; Intestinal permeability; Single-pass intestinal perfusion; P-glycoprotein; RP-HPLC

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INTRODUCTION

Metformin (dimethylbiguanide), N,N-dimethylimidodicarbonimidic diamide (Figure 1) is an oral antihyperglycaemic agent used in the management of non-insulin-dependent diabetes mellitus (NIDDM). It reduces blood glucose levels predominantly by improving hepatic and peripheral tissue sensitivity to insulin without affecting the secretion of this hormone. Metformin also appears to have potentially beneficial effects on serum lipid levels and fibrinolytic activity, although the long-term clinical implications of these effects are unclear^[1]. Gastrointestinal absorption of metformin is incomplete, 20%-30% of an oral dose is recovered in the faeces^[2]. Absorption is estimated to be complete within 6 h of administration and is presumably confined to the upper part of the intestine^[2,3]. Metformin is poorly absorbed from the stomach and the delivery process highly correlated with the rate-limiting factor for metformin absorption from the duodenum, while the whole intestine is necessary for sufficient absorption of drugs^[3]. Proportionately more drugs are absorbed after 0.5 g dose than 1.5 g dose, possibly because of the involvement of an active, saturable absorption process^[3]. In clinical practice,

metformin is an effective antihyperglycaemic agent with additional beneficial effects on metabolic and fibrinolytic variables, both of which can be used as monotherapy or in combination with other antihyperglycaemic agents^[1].

The SPIP technique is performed in anesthetized rats whereby a section of intestine is isolated and perfused with a solution of the compound of interest. In the SPIP experimental procedure modifications can be made to the flow rate, length of perfused intestine, and concentration of the compound of interest thus giving the investigator exquisite control of the factors influencing the intestinal absorption of a chemical. In the most basic SPIP protocol the compound of interest is monitored in the perfusate only but not in the blood. Loss of compound, as determined by the difference between the inlet and outlet concentrations, is attributed to absorption but only after preliminary studies rule out other possible factors. The preliminary studies may consist of stability studies in the buffer (unperfused and blank perfused buffered) and homogenates of the intestine.

In vivo rat studies have also been performed in an attempt to study the role of intestinal P-gp in drug absorption and metabolism. P-gp, a plasma membrane protein of about 170 kDa, has been demonstrated in many normal tissues, including intestinal cells^[4,5]. P-gp in the gut wall acts as an efflux transporter of certain drugs and studies in several species including the rat indicate that P-gp may play an important role in limiting drug absorption^[6,7]. P-gp, a member of the ATP-binding cassette transporter superfamily (ABCB1) and is located on the apical membrane of intestinal enterocytes where it can actively efflux drugs from the cells back into the intestinal lumen^[8]. An increasing number of drugs, including HIV protease inhibitors like indinavir, zidovudine, saquinavir and anti-cancer drugs like paclitaxel, docetaxel, etc, have been reported to be substrates for P-gp^[9]. Verapamil, a P-gp substrate, is a competitive inhibitor of intestine P-gp in the rat^[10], and is used as a tool for P-gp inhibition^[10].

The aim of this study was to characterize and classify the intestinal permeability of metformin in rats using SPIP model, and to predict the intestinal absorption mechanism of metformin in humans as well as to investigate whether metformin is a substrate for P-gp.

MATERIALS AND METHODS

Instruments

The high performance liquid chromatography (HPLC) system consisting of a Shimadzu LC-6A auto solvent delivery module pump was purchased from (Shimadzu, Kyoto, Japan). Gilson 118 UV spectrophotometric detector, SCL-6A system controller, CTO-6A column oven, CKChrom chromatograph data system, syringe pump (BT01-YZ1515-B) were produced of Tianjin Xieda Electronic Co. Ltd (Tianjin, China). Homogenizer was from Shanghai Jinda Biochemical Instrument Factory (Shanghai, China). Heating operating table was from Shanghai No.1 Medication Store (Shanghai, China). Analytical balance (TG328A) and electronic balance (JA2003) were obtained from Shanghai Balance Instrument Factory (Shanghai, China). High speed table centrifuge (TGL-16) was purchased from Shanghai

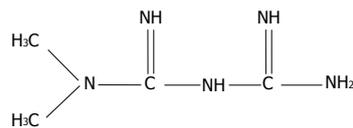


Figure 1 Structure of metformin (dimethylbiguanide).

No.6 Medical Instruments Factory (Shanghai, China). Constant temperature water bath chamber was a produced of Beijing Xicheng Medical Instruments Factory (Beijing, China).

Drugs and reagents

Metformin (purity: 99.5%) and verapamil (purity: 99.5%) were produced by Department of Pharmaceutical Preparation, Tianjin Institute of Pharmaceutical Research (Tianjin, China). Doxofylline was produced by Research Center for New Drug Evaluation, Tianjin Institute of Pharmaceutical Research (Tianjin, China). Urethane was purchased from Beijing Chemical Engineering Plant (Beijing, China). Phenol red and ammonium acetate (analytical grade) were purchased from Tianjin Chemical Reagent No.1 Plant (Tianjin, China). Ion pair reagent, 1-octanesulfonic acid sodium salt (IPR-B₈, 0.25 mol/L) was purchased from Tianjin Chemical Reagent No.2 Plant (Tianjin, China). Methanol (HPLC grade) was purchased from Tianjin Concoct Chemical Reagent Company (Tianjin, China).

Animals

Male Wistar rats weighting 180-230 g were purchased from Center of Experimental Animals, Tianjin Institute of Pharmaceutical Research (Certificate No. 20050110), Tianjin, China. Animals were acclimated for at least 5 d before the experiments and housed in cage (5 each) under constant temperature (22 ± 2°C) with free access to food and drinking water. Animals were fasted overnight before use either in the SPIP study or for harvesting intestinal tissue to prepare homogenate preparation.

Perfusion solution

Krebs-Ringe buffer solution^[11] (K-R buffer solution containing 7.8 g NaCl, 0.35 g KCl, 1.37 g NaHCO₃, 0.02 g MgCl₂, 0.22 g NaH₂PO₄ and glucose in 1.48 g/1000 mL purified water) was used as blank perfusion solution. Phenol red (20 µg/mL) and doxofylline (20 µg/mL) were added to perfusion solution as non-absorbable marker and internal standard, respectively. Perfusion solution containing 10 µg/mL, 50 µg/mL, and 200 µg/mL metformin respectively, 20 µg/mL phenol red and 20 µg/mL doxofylline was used. In addition, 400 µg/mL verapamil was added to perfusion solution containing 50 µg/mL metformin to achieve solution containing P-gp inhibitors.

Collection of rat small intestinal fluid and Preparation of homogenates

After an overnight fast, animals were anesthetized with an intraperitoneal (i.p.) injection of urethane (0.7 mL/100 g). Upon verification of the loss of pain reflex, a midline abdominal incision of 3-4 cm was made and a 10-15 cm intestinal segment of interest (duodenum, jejunum and

ileum) was isolated and cannulated at both ends with plastic tubing. The segment was first rinsed with 37°C saline to clear the segment, then blank K-R buffer solution (without drug) was pumped into the segment at a constant flow rate of 0.2 mL/min. Blank perfused solution was collected at the outlet and used to prepare metformin solution (10, 50, 200 µg/mL) with doxofylline (20 µg/mL) for stability studies of metformin.

To prepare intestinal homogenates, animals were anesthetized as the above procedure, a 10-15 cm intestinal segment of interest (duodenum, jejunum and ileum) was isolated and removed. The excised intestinal segment was washed with ice-cold buffer and the mucosa was removed from the intestine by scraping the intestine. The intestinal mucosa was weighed and then homogenized with enough ice-cold blank K-R buffer solution to make a 20% w/v homogenate using a homogenizer. The homogenate was then centrifuged for the resultant supernatant, which was used to prepare metformin solution (10, 50, 200 µg/mL) with doxofylline (20 µg/mL) for stability studies of metformin.

Single pass intestinal perfusion experiment

SPIP studies were performed using established methods as previously described^[12,13]. Male Wistar rats weighing 180 g to 230 g were fasted overnight before the perfusion experiment with free access to tap water only. The rats were anesthetized with i.p. injection of urethane (0.7 mL/100 g) and placed on a heating operating table to maintain a body temperature of 37°C. Upon verification of the loss of pain reflex the abdomen was opened by a 3-4 cm midline longitudinal incision of and a 10-15 cm intestinal segment (duodenum, jejunum and ileum) was isolated and cannulated at both ends with plastic tubing. The segment was first rinsed with 37°C saline to clear the segment, and approximately 10 cm of the inlet tubing was placed inside the abdominal cavity to achieve an inlet perfusion solution at 37°C. Drops of saline were added onto the surgical area, which was then covered with wet pledget to avoid loss of fluid. The experiment was initiated by rapidly filling the segment with perfusion solution and the time was set zero with the immediate start of the perfusion. The perfusion rate was 0.2 mL/min. After approximately 30 min, when steady-state was achieved, the outlet perfused samples were collected on ice at 15 min intervals up to 105 min. Samples were frozen immediately and stored at -20°C. At the end of the experiment, the perfused intestinal segment was measured without stretching.

Effects of drug concentration and different intestinal site on intestinal absorption

Thirty rats were divided into 6 groups ($n = 5$) in the present study. SPIP was performed in different intestinal segments (duodenal, jejunal and ileal segment) to test if the intestinal absorption of metformin could exhibit the characteristics of intestinal site dependence. Each intestinal segment was isolated as follows: duodenum segment beginning from pylorus, jejunum segment beginning from 25 cm away from pylorus, while ileum segment the beginning at the site 20 cm upwards caecum. The above seg-

ments were perfused with solution containing 50 µg/mL metformin and 20 µg/mL phenol red. The experimental operation was carried out as described above.

Perfusion solutions of different drug concentrations were perfused to investigate the intestinal absorption mechanism of metformin. Duodenum segment was selected as the perfused segment, perfusion solutions containing 10, 50, 200 µg/mL metformin as well as 20 µg/mL phenol red were perfused to test if the permeability values exhibit concentration-dependent changes.

Effects of P-gp on intestinal absorption

Duodenum segment was selected for perfusion, 400 µg/mL verapamil was added to the perfusion solution (containing 50 µg/mL metformin and 20 µg/mL phenol red) as an inhibitor of P-gp exiting along the gastrointestinal track, then the changes of metformin absorption were determined to find out if the intestinal absorption of metformin was affected by P-gp exiting along the gastrointestinal track.

Sample analysis

The absorption and stability of samples were analyzed by the HPLC system at UV 250 nm with the oven temperature at 40°C. Analytes were separated on a Diamonsil™ ODS C₁₈ column (5 µm, 200 mm × 4.6 mm I.D.). The mobile phase was made up of methanol: 20 mmol/L NH₄AC (containing 1% 0.25 mol/L IPR-B₈) = 30:70 (V:V). The flow rate was 1.0 mL/min. Doxofylline (20 µg/mL) was used as an internal standard. Retention time for metformin, doxofylline, phenol red was 5.1 min, 7.2 min and 11.6 min respectively. All sample peaks were well separated (Figure 2).

Data analysis

Calculations were based on outlet perfusate steady-state concentrations achieved after approximate 30 min. The steady-state intestinal effective permeability (P_{eff} , cm/s) was calculated according to a parallel tube model^[14-16]:

$$P_{eff} = [-Q_{in} \cdot \ln (C_{out}/C_{in})] / A \quad (1)$$

Where Q_{in} is the perfusion flow rate (0.2 mL/min), A is the mass transfer surface area within the intestinal segment assumed to be the area of a cylinder ($2\pi rL$) with the length (L) (measured after 45 min) and radius (r) of 0.18 cm^[15,17], C_{in} and C_{out} are the inlet and fluid-transport-corrected outlet solution concentrations, respectively. The latter was corrected by multiplying the inlet concentration with $[\text{phenol red}]_{in}/[\text{phenol red}]_{out}$.

Standard curve

Metformin stock solution (5, 10, 20, 50, 100, 200 µL) of and phenol red stock solution (10, 20, 30, 40, 50, 60 µL) were precisely drawn, then diluted to 1 mL with K-R buffer solution to make corresponding solutions containing metformin and phenol red 5 and 10, 10, 20, 30, 40, 50, 60 µg/mL phenol red, respectively. The solutions were then injected for determination of calibration curve. The regression curve for metformin and phenol was $Y = -0.00634 + 0.00599X$ ($r = 0.9998$) and $Y = -0.00814 + 0.00870$ ($r = 0.9995$), respectively, indicating good linear correlations.

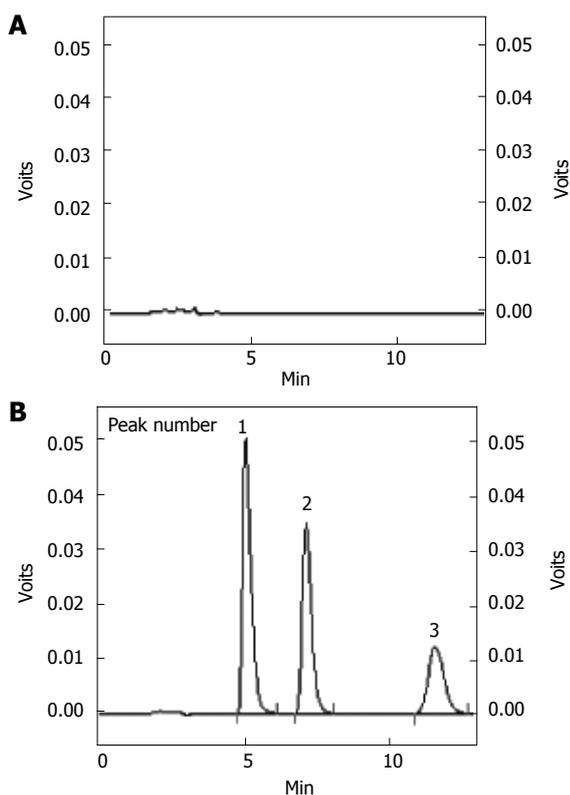


Figure 2 Representative chromatograms of blank (A) and *in situ* perfusion (B) samples containing metformin, doxofylline and phenol red (1: metformin; 2: doxofylline; 3: phenol red).

Accuracy and precision

The intraday and interday precision as well as the accuracy were determined with quality control samples, and five replicates each were analyzed on three consecutive days. Three concentrations were chosen from the high medium and low range of the standard curve (10, 50, 200 $\mu\text{g}/\text{mL}$ for metformin and 20, 40, 60 $\mu\text{g}/\text{mL}$ for phenol red). Precision was expressed as the relative standard deviation (RSD%). Accuracy was expressed as the mean relative error (RE%). A precision (RSD%) less than or equal to 15% and a accuracy (RE%) less than or equal to 15% were accepted. The data are summarized in Table 1.

Recovery

The absolute recovery was determined at three concentrations (10, 50, 200 $\mu\text{g}/\text{mL}$ for metformin, and 20, 40, 60 $\mu\text{g}/\text{mL}$ for phenol red) ($n = 6$ at each concentration). Results showed that the absolute recovery of metformin at these three concentrations ranged from $97.6 \pm 3.73\%$ to $101.6 \pm 3.14\%$, with RSD% less than 3.83%.

Stability studies

The stability study consisted of stability studies in the K-R buffer solutions (unperfused and blank perfused) as well as in homogenates of the intestine. The stability of metformin was based on the decrease of the parent compound after 2 h incubation in 37°C water bath as quantitated by HPLC. The stability was determined at concentration of 50 $\mu\text{g}/\text{mL}$ metformin, and 40 $\mu\text{g}/\text{mL}$ phenol red, $100.8 \pm 0.74\%$ and $108.1 \pm 3.22\%$ metformin

Table 1 Accuracy and precision data for assays of metformin and phenol red in perfused solution

Concentration ($\mu\text{g}/\text{mL}$)	Intra-d ($n = 5$)		Inter-d ($n = 5$)	
	Accuracy Mean RE %	Precision CV %	Accuracy Mean RE %	Precision CV %
Metformin				
10	-0.9	3.2	-3.5	4.2
50	1.5	1	6.8	6.3
200	0.9	2.1	10.5	0.7
Phenol red				
20	-2.4	3.8	7.8	6.4
40	-1.4	2.1	6.6	6.1
60	1.6	3.1	14.4	3.3

remained after perfusion with unperfused and perfused blank K-R buffer for 2 h. For the perfusion with intestinal homogenate, 2 h incubation at least $87.5 \pm 0.69\%$ metformin remained in the homogenate ($101.9\% \pm 1.74\%$, $87.5\% \pm 0.69\%$ and $90.0\% \pm 1.75\%$ for duodenal, jejunal, ileal homogenate, respectively). Results demonstrated that in the stability study, metformin was sufficiently stable ($> 80\%$ remaining) for the duration of the SPIP experiment (90 min) in K-R buffer solution, perfused K-R buffer solution and intestinal homogenates.

Statistical analysis

The results reported were expressed as means \pm SD. The statistical difference between treatment groups was evaluated using analysis of variance (ANOVA) and the identification of significance was carried out with LSD *post hoc* test. $P < 0.05$ was considered statistically different.

RESULTS

Intestinal permeability of metformin was determined in rat intestine segments (duodenum, jejunum and ileum) using *in situ* single-pass perfusion technique and the samples were analyzed by the proposed method. Effective permeability values were calculated from the steady-state concentrations of compounds in the perfusate collected from the outlet. Steady-state was confirmed by the ratio of the outlet to inlet concentrations (corrected for water transport) *versus* time. Representative results are shown in Figure 3. The effective permeability values for each intestinal segment at each concentration are plotted in Figure 4.

Effects of drug concentration and different intestinal site on intestinal absorption

Intestinal absorption site dependent changes in permeability were found in the jejunum and ileum where the permeability at 50 $\mu\text{g}/\text{mL}$ of metformin [$(3.26 \pm 0.73) \times 10^5$ and $(2.96 \pm 0.36) \times 10^5$ cm/s, respectively] was significantly different from those in the duodenum at the same concentration [$(4.51 \pm 1.08) \times 10^5$ cm/s, $P < 0.05$]. Furthermore, concentration dependent changes in permeability were also found in the duodenum where the permeability at 200 $\mu\text{g}/\text{mL}$ of metformin [$(2.70 \pm 0.63) \times 10^5$ cm/s] was significantly different from that at 10 $\mu\text{g}/\text{mL}$ of metformin $(4.71 \pm 0.86) \times 10^5$ cm/s and 50 $\mu\text{g}/\text{mL}$ of metformin $(4.51 \pm 1.08) \times 10^5$ cm/s, $P < 0.05$.

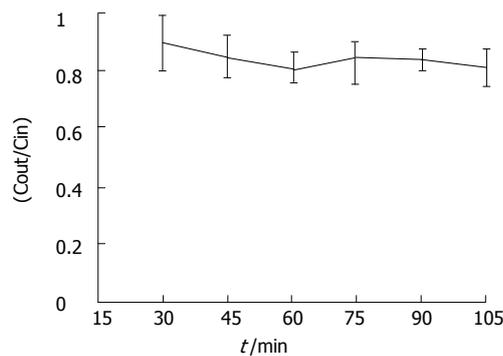


Figure 3 Approach to steady-state for metformin existing in the intestinal perfusate ($n = 5$).

Effects of P-gp on intestinal absorption

The P-gp efflux inhibitor, verapamil did not affect the measured duodenum P_{eff} for metformin at 50 $\mu\text{g/mL}$. No significant increase in permeability was found after co-perfusion with verapamil (400 $\mu\text{g/mL}$) in duodenum [$(4.51 \pm 1.08) \times 10^5$ cm/s for metformin at 50 $\mu\text{g/mL}$ and $4.53 \pm 1.75 \times 10^{-5}$ cm/s for co-perfusion with 400 $\mu\text{g/mL}$ verapamil].

DISCUSSION

Drugs can cross membranes either by passive processes or by mechanisms involving the active participation of components of the membrane. In the former the drug molecule usually penetrates by passive diffusion along a concentration gradient by virtue of its solubility in the lipid bilayer. The two most common ways for the absorption of drugs are passive transfer by diffusion across the liquid membranes and passive diffusion through the aqueous pores at the tight junctions between cells, which are referred to as transcellular and paracellular absorption, respectively. Transcellular absorption is the predominant pathway for more lipophilic molecules^[18]. In contrast, the paracellular route of absorption is limited to small, hydrophilic compounds^[19]. Active transport (carrier-mediated membrane transport) is characterized by selectivity, competitive inhibition by congeners, a requirement for energy, saturability, and movement against an electrochemical gradient. Intestinal transport of a compound commonly employs a combination of several mechanisms. The relative importance of each pathway depends on a variety of physicochemical and physiological conditions.

The intestinal permeability is the propensity of a compound to move across the epithelial barrier of the intestine. *In vitro* and *in situ* absorption models, such as *in situ* perfusion of rat intestine, Caco-2 cell monolayer model, and excised intestinal segments in the Ussing chamber, are commonly used to investigate transport mechanism, classify permeability and *in vivo* absorption of drugs in humans^[20,21]. Comparisons between human P_{eff} data and these preclinical permeability models showed that they could be used to investigate and classify passive transport with a high accuracy^[22]. Recently, a GI-transit-absorption model (GITA) has been developed to analyze and predict drug absorption, and there is some evidence that this model is

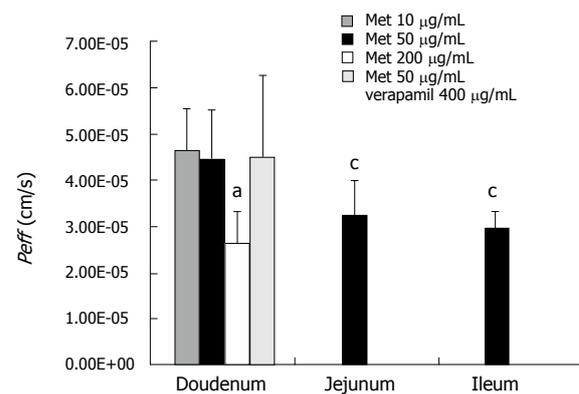


Figure 4 Effective permeability, P_{eff} (cm/s) of metformin using single-pass intestinal perfusion. For each region, P_{eff} of metformin was determined at 50 $\mu\text{g/mL}$. For duodenum segment, P_{eff} of metformin was determined at 10, 50, 200 $\mu\text{g/mL}$, as well as 50 $\mu\text{g/mL}$ with addition of verapamil. The data were presented as mean \pm SD ($n = 5$ in each group). ^a $P < 0.05$ vs 10 and 50 $\mu\text{g/mL}$ metformin in duodenum; ^c $P < 0.05$ vs 50 $\mu\text{g/mL}$ metformin in duodenum.

very useful for estimating the absorption kinetics of drugs with various characteristics as well as the human data^[23].

Among the various models, cell-based assays using Caco-2 and MDCK cell lines are commonly used to predict passive absorption and the results obtained are greatly affected by experimental parameters such as pH^[24]. In contrast, *in situ* approaches provide experimental conditions closer to what is encountered following oral administration, with a lower sensitivity to pH variations due to a preserved microclimate above the epithelial cells^[25,26]. These techniques maintain an intact blood supply to the intestine, and can be used to estimate the impact of clearance pathways, such as enzymes and transporters, that are present in the gut. Moreover, drug permeability^[27], expression of drug metabolizing enzymes and transporters have been shown to vary along the intestinal tract^[28-30], which can be investigated using intestinal perfusion of the various regions. In addition, it was recently reported that oral drug absorption in rats and humans is very similar^[31]. Thus, it is likely that the intestinal perfusion conducted in rats may give a better prediction of the fraction of oral dose absorbed in humans than in *in vitro* models.

The SPIP procedure assumes that loss of drug during the perfusion is due to permeation of the intestine. Therefore, determination of the non-absorptive loss of perfused drug is required. Results of preliminary studies have confirmed that the loss of metformin in the SPIP study could be attributed to the intestinal absorption, and the confidence of the calculated permeabilities is high. The intestinal effective permeability represents a direct measurement of the local absorption rate and reflects the transport velocity across the epithelial barrier, expressed as centimeter per second^[32]. In the present study, SPIP was performed in three different intestinal segments to test if the intestinal absorption of metformin exhibited site-dependent changes. The results indicated that metformin could be absorbed along the whole intestine with the main absorption site at duodenum, contributing to the drug absorption. Further comparison of permeability values among high, medium and low concentrations in the duodenal segment demonstrated a concentration-dependent

change, exhibiting a decrease in permeability at the highest drug concentration. The result suggested that intestinal absorption of metformin was *via* passive and transcellular mechanism involving an active saturable process, or *via* a membrane transport protein in the gut wall, especially in the duodenum.

The present study also demonstrated that metformin could not be used as a substrate for rat intestinal P-gp because permeability values of metformin in the duodenum segment at 50 µg/mL were not increased by co-perfusion of the P-gp inhibitor verapamil, suggesting that metformin cannot be efficiently transported by P-gp in the gut wall, however, a saturable process may be involved in the intestinal transport of metformin. Since the SPIP experimental protocol cannot completely elucidate transport mechanism (i.e. inability to conduct directional transport studies), further studies with cultured cells or isolated tissue are necessary to identify the particular reason for the concentration dependent changes in permeability. Such studies would permit determination of both influx and efflux permeabilities. Experiments with cells transfected with the gene coding for a particular transport would further identify the specific protein involved in the carrier-mediated process.

Effective intestinal permeability estimated from *in situ* perfused rat intestine has been shown to correlate well with the extent of *in vivo* absorption in humans after oral administration of carrier-mediated absorbed drugs^[33]. It was recently reported that special care must be taken for drugs with a carrier-mediated transport mechanism^[22]. Furthermore, although *in situ* perfusion technique is time consuming, it provides a greater correlation with intestinal absorption in humans than Caco-2 and MDCK cell lines. Thus human intestinal permeability values of metformin may be predicted from permeability values obtained in this rat SPIP experiment according to^[16]:

$$P_{eff,man} = 3.6 \cdot P_{eff,rat} + 0.03 \cdot 10^{-4} \quad (2)$$

Based on the predicted permeability value in humans, the fraction absorbed in humans ($f_{a,min}$) can further be predicted from rats for carrier-mediated absorbed drugs by^[34]:

$$f_{a,man} = 1 - e^{-[2 \cdot P_{eff,man} \cdot t_{res} / r \cdot 2.8]} \quad (3)$$

Where t_{res} is the average small intestine transit time, r is radius in humans (assumed to be 3h and 1.75 cm, respectively).

Using equation (3) and the lowest permeability value of metformin obtained in this study (2.70×10^{-5} cm/s in the duodenum at 50 µg/mL of metformin), the permeability value of metformin in humans was estimated to be 1.0×10^{-5} cm/s. Then applying equation to metformin for the lowest permeability value in humans in this study gave an estimated $f_{a,man}$ of 74%, while for the highest one gave an estimated $f_{a,man}$ of 90%, which is in accordance with the previously reported values^[2,3].

The successful development of oral drug delivery formulations requires characterization of the intestinal absorption. Before the preparation for oral administration of extended-release and controlled-release system and targeting drug release system in gastrointestinal track, characteristics of drug absorption and transfer in the gastrointestinal track should be carefully studied and well understood. In general, appropriate candidates for extended-

release and controlled-release preparations are molecules that have poor colonic absorption but are characterized by better absorption properties at the upper parts of the gastrointestinal tract^[16]. Results of the present study showed that metformin could be absorbed in the whole human intestine, with the main absorption site at duodenum. However, it was reported that absorption of metformin in human intestine is incomplete, 20%-30% of an oral dose can be recovered in the faeces. Furthermore, it has been reported that oral absorption of the drug is slower than that of elimination^[35]. Therefore special care should be paid to the drug content and drug administration times (maybe 2 times/d) when preparing extended-release and controlled-release formulations in order to maintain the effective blood concentration. The common side effects of metformin therapy could be concluded as gastrointestinal symptoms, such as abdominal discomfort, nausea, and diarrhea that especially occur during the initial weeks of treatment^[36]. Employing of extended-release technique could reduce the direct drug stimulation to the gastrointestinal tract, and may reduce the above gastrointestinal symptoms due to the lower peak exposure of intestinal tissue to the drug.

In conclusion, intestinal permeability of metformin is concentration- and site- dependent. Fraction of the oral dose absorbed after oral administration is 74%-90% along the whole human intestine. Intestinal absorption of metformin may be *via* passive and transcellular mechanism involving an active, saturable process, or *via* a membrane transport protein in the gut wall, especially in the duodenum. The observed intestinal permeability values obtained in the SPIP model fit well the pattern observed in previous permeability studies for other structural different drugs in rat intestine.

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Inhibitory effect of Fuzheng Yiliuyin in combination with chemotherapeutics on human gastric carcinoma cell strain

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Abstract

AIM: To study the inhibitory effects of Fuzheng Yiliuyin (Decoction for Suppressing Tumors by Strengthening the Body Resistance) in combination with chemotherapeutics on human gastric carcinoma cell strain.

METHODS: Fuzheng Yiliuyin (ZY) combined with various kinds of chemotherapeutics was put into two kinds of cultivated human gastric carcinoma cell strains, then its inhibitory effects on human gastric carcinoma cell strains were determined by the MTT method. Flow cytometer was used to assay the apoptosis rate, and the ultrastructure of gastric carcinoma cells was observed under transmission electron microscope.

RESULTS: Obvious apoptosis was seen in gastric carcinoma cells after treatment with ZY for 72 h. ZY and chemical drugs had synergistic inhibition effects on the cultivated gastric carcinoma cells, but the effects were different on various cell strains. The inhibitory effects of ZY could be strengthened by cytotoxic action and apoptosis. ZY combined with fluorouracil, etoposide and cisplatin (EFP) chemotherapeutics had better inhibitory effects on SGC-7901, while ZY combined with EFP or with DDP chemotherapeutics had better inhibitory effects than other drugs on MGC-803.

CONCLUSION: ZY induces apoptosis and inhibits the growth of gastric carcinoma cells. ZY has the synergistic function of chemotherapeutics.

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Key words: Human gastric carcinoma cell strain; Traditional Chinese medicine; Chemotherapeutics

INTRODUCTION

Fuzheng Yiliuyin (ZY) has been used for 30 years to treat gastric carcinoma in our clinic. ZY is prepared from eleven herbs: milkvetch root, bighead atractylodes rhizome, Chinese Thorowax root, common Burreed rhizome, Zedoary, Radix Notoginseng, Dandelion, spreading Hedyotis herb, Agrimony, Tamariskoid spikemoss herb and Glycyrrhizia. Most of these herbs can inhibit cell proliferation, induce apoptosis and have anti-cancer effects^[1-4]. *In vitro* experiment has confirmed that Fuzheng Yiliuyin is effective in inhibiting proliferation of gastric cancer cells and induces apoptosis^[5]. Further double-blind placebo-controlled trial of chemotherapy plus Fuzheng Yiliuyin/placebo in advanced stage gastric cancer showed that ZY has anti-gastric cancer effects with lower side effects^[6]. Traditional Chinese medicines TCM could increase the effects and decrease the side effects of chemotherapeutics. However, whether TCM can increase all of the therapeutic effects of chemotherapeutics and whether the mechanism is the same remain unclear.

MATERIALS AND METHODS

Patients

The human gastric mucinous adenocarcinoma cell line (MGC-803) with lower differentiation was obtained from the Center of Molecular Biology of Xi'an Jiaotong University, China. The human gastric adenocarcinoma cell line (SGC-7901) with lower metastasis was obtained from Xijing Hospital of the Fourth Military Medical University, China. Fuzheng Yiliuyin (ZY) extract contains 2 g/L crude herbs, which was produced by Xi'an Jiaotong University. Fluorouracil (5-FU, Shanghai Pharmaceutical Factory), etoposide (Vp-16 Lianyungang Pharmaceutical Factory), cisplatin (DDP, Dezhou Pharmaceutical Factory), adriamycin (Pharmacia and Upjohn), annexin-V-FITC and PI (Roche), EDTA and MTT (Sigma) were used in the study.

Cell culture

Cells were cultured in RPMI1640 medium (pH 7.2-7.4)

Table 1 Effects of ZY in combination with chemotherapeutics on SGC-7901 MGC-803 cell proliferation (mean \pm SD)

Group	SGC-7901			MGC-803		
	24 h	48 h	72 h	24 h	48 h	72 h
ZY	124 \pm 7.9	17.4 \pm 3.1	201 \pm 5.5	8.9 \pm 4.5	11.8 \pm 5.4	14.8 \pm 2.7
ZY + Vp-16	^a 51.2 \pm 4.2	^a 56.9 \pm 4.8	^a 62.3 \pm 3.9	^a 36.9 \pm 7.2	^a 48.9 \pm 5.2	^a 58.8 \pm 4.6
ZY + ADM	^a 46.5 \pm 7.2	^a 50.1 \pm 3.2	^a 55.4 \pm 4.8	^a 34.5 \pm 8.1	^a 43.6 \pm 3.7	^a 50.7 \pm 6.4
ZY + 5-Fu	^a 57.8 \pm 2.6	^a 61.9 \pm 6.3	^a 69.9 \pm 8.4	^a 40.7 \pm 6.6	^a 50.8 \pm 4.7	^a 55.7 \pm 3.2
ZY + DDP	^a 53.6 \pm 2.4	^a 58.4 \pm 1.7	^a 67.4 \pm 5.7	^a 70.2 \pm 3.5	^a 80.1 \pm 3.6	^a 84.3 \pm 7.5
ZY + EAP	^a 61.2 \pm 3.3	^a 68.5 \pm 3.9	^a 71.5 \pm 3.4	^a 73.8 \pm 4.6	^a 72.2 \pm 4.1	^a 74.9 \pm 3.4
ZY + EFP	^a 79.4 \pm 2.8	^a 83.3 \pm 4.8	^a 87.5 \pm 4.3	^a 82.6 \pm 7.8	^a 87.5 \pm 3.1	^a 89.9 \pm 4.8

^a*P* < 0.05 vs 24 h, ^b*P* < 0.05 vs 48 h, ^c*P* < 0.05 vs 72 h, ^d*P* < 0.05 vs 24 h, ^e*P* < 0.05 vs 42 h, ^f*P* < 0.05 vs 72 h.

containing 100 mL/L foetal calf serum (FCS), penicillin and streptomycin at 37°C in a humidified (95%) incubator containing 95% air and (50 mL/L) 5% CO₂. Cells were routinely sub-cultured using 2.5 g/L trypsin/ethylenedinitrile tetra-acetic acid (EDTA) solution.

MTT assay for determination of cell growth

Cell proliferation was determined by counting viable cells over time using trypan blue exclusion as the basis of viability. The logarithmically growing SGC-7901 and MGC-803 cells were plated at a density of 5×10^7 cells/L per well into a 96-well plate. After 24 h, the cells were treated with 1 mg/L ZY in combination with 5-Fu, Vp-16, ADM, DDP, EFP and EAP, respectively for 24, 48, and 72 h. Control wells were treated with RPMI1640 medium alone. Then 20 μ L MTT (5 g/L) was added to each well and incubated for an additional 4 h. After the culture media were discarded, 0.15 mL DMSO was added and vibrated for 10 min. The absorbance was measured at 570 nm using a Model 550-microplate reader. The inhibitory rates (IR) were calculated according to the following formula:

$$\text{IR (\%)} = (1 - \text{absorbance of the treated wells}) / (\text{absorbance of the control wells}) \times 100\%$$

Flow cytometry of apoptosis by annexin-V and PI double-staining

At the end of treatment, the cells were harvested by trypsin-EDTA solution to produce single cell suspension. Annexin-V and PI double staining kit (Roche) were used to assess apoptosis. The cells were analyzed by flow cytometry. Early apoptotic cells were localized in the lower right quadrant of a dot-plot graph using annexin-V-fluorescein and PI.

Cell morphology observation

The SGC-7901 and MGC-803 cells were separately incubated with 1 mg/L ZY for 72 h. The culture medium was discarded, the cells were collected by gently scraping and washed three times in ice-cold PBS, then fixed with a solution of 2% formaldehyde and 3% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer (pH 7.4) for 1 h. The fixed cells were washed three times in cacodylate buffer (pH 7.2) containing 0.2 mol/L sucrose, fixed with 1% osmic acid in 0.3 mol/L cacodylate buffer, dehydrated in

Table 2 Effects of ZY in combination with chemotherapeutics on SGC-7901 MGC-803 cell apoptosis rates (mean \pm SD)

	SGC-7901			MGC-803		
	alive	necrosis	apoptosis	alive	necrosis	apoptosis
ZY	78.7 \pm 0.6	6.6 \pm 4.3	14.5 \pm 5.4	84.4 \pm 3.1	4.5 \pm 0.6	11.2 \pm 1.8
ZY + Vp-16	38.5 \pm 2.5	46.1 \pm 6.2	15.3 \pm 2.1	42.1 \pm 1.5	32.4 \pm 7.9	25.8 \pm 5.2
ZY + ADM	44.6 \pm 3.5	26.7 \pm 2.5	19.9 \pm 4.7	49.4 \pm 5.2	29.8 \pm 3.3	20.7 \pm 5.1
ZY + 5-Fu	29.6 \pm 4.1	44.7 \pm 9.9	25.6 \pm 1.2	44.3 \pm 8.2	30.5 \pm 7.4	25.3 \pm 4.6
ZY + DDP	32.3 \pm 11.7	63.6 \pm 10.6	4.1 \pm 3.0	16.1 \pm 7.5	60.9 \pm 8.1	23.0 \pm 0.6
ZY + EAP	27.6 \pm 5.8	55.5 \pm 10.5	16.8 \pm 7.4	25.0 \pm 2.9	65.4 \pm 5.8	9.6 \pm 2.3
ZY + EFP	13.1 \pm 2.7	66.3 \pm 12.0	20.7 \pm 6.6	11.1 \pm 3.6	67.8 \pm 4.1	20.9 \pm 4.9
control	92.1 \pm 6.3	6.6 \pm 8.4	1.3 \pm 0.2	94.3 \pm 0.2	4.6 \pm 0.8	1.2 \pm 0.5

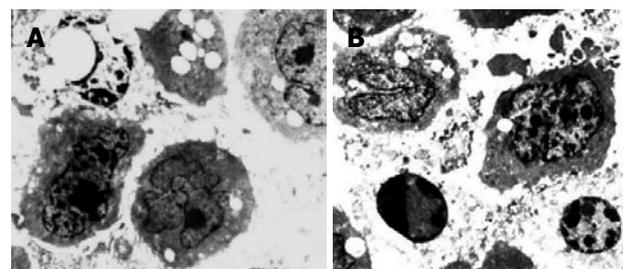


Figure 1 Morphological change of apoptosis in SGC-7901 (A) and MGC-803 (B) cells after treated with ZY.

a graded series of acetone, and embedded in epoxy resin. Ultrathin sections were cut, stained with uranyl acetate and lead citrate, and assessed by electron microscopy

Statistical analysis

Data were expressed as mean \pm SD. Analysis of data was performed using one-way ANOVA. *P* < 0.05 was considered statistically significant.

RESULTS

SGC-7901 and MGC-803 cells were treated with various chemotherapeutics. Cell viability was determined by MTT assay. ZY increased the inhibitory effects of various chemotherapeutics on both kinds of cells in a time-dependent manner. ZY in combination with EFP had a better effect on SGC-7901 cells, while ZY in combination with DDP and EFP had a better effect on MGC-803 cells (*P* < 0.05).

After SGC-7901 and MGC-803 cells were exposed to ZY in combination with various chemotherapeutics for 72 h, annexin-V and PI double-staining FCM analysis showed that the apoptosis rates of SGC-7901 and MGC-803 cells were quite different after treated with different drugs. ZY increased anticancer effects of chemotherapeutics by directly enhancing the toxic effects or by inducing apoptosis (Table 2).

In order to confirm the cell apoptosis induced by treatment with ZY, the morphological changes of apoptosis were evaluated by transmission electron microscopy. Changes were observed in SGC-7901 cells (Figure 1A) and MGC-803 cells (Figure 1B) after treated with ZY, such as blebbing and loss of microvilli, vacuolation in cytoplasm, condensation of cytoplasm and nuclei, and fragmented

chromatin, which provided further evidence for the induction of apoptosis as a consequence of ZY treatment.

DISCUSSION

Gastric cancer is a common malignant tumor of the alimentary tract and its incidence is among the three leading kinds of neoplasm in different regions of China^[7,8]. Conventional treatments for advanced gastric cancer including extended resection, radiotherapy and chemotherapy have little influence on the improvement of patients' survival. Combinations such as FAMTX, ELF, EAP and EFP, produce an overall response rate of about 20% to 50%^[9-12]. Though combination chemotherapy for local advanced gastric cancer is effective, it is often associated with severe side effects, including fatal outcome.

Traditional Chinese herbal medicines can enhance the efficacy and reduce the toxicity of chemotherapy and radiotherapy by improving anticancer host defense mechanism and inducing apoptosis^[13-19]. The use of Chinese herbal medicine in combination with radiotherapy or chemotherapy is a better procedure in the treatment of advanced cancer.

This study showed that Chinese herbal medicines could increase the effects of chemotherapy on gastric cancer, but the cytostatic effect of it is different between different differentiation cells. This is why the same Chinese herbal medicine in combination with the same chemotherapy is effective in some patients and less effective in other patients. Though combined chemotherapeutic agents may show a better sensitivity than a single agent, and various drugs show various effects on various alimentary tract tumors^[20]. In this study, ZY in combination with a single chemotherapeutic agent or with several chemotherapies had the similar inhibitory effects on the same cells, suggesting that individual therapy should be performed based on the type and differentiation degree of tumors. Individual chemosensitivity is essential to chemotherapy for gastric cancer^[21].

Apoptosis plays a critical role in tumor initiation, progression, as well as in cancer therapy^[22]. In the present study, we investigated the inhibitory effects of ZY plus chemotherapy on gastric carcinoma SGC-7901 and MGC-803 cells. ZY caused not only cellular necrosis but also induced apoptosis and inhibited the proliferation of gastric cancer cells, indicating that TCM in combination with chemotherapy can induce apoptosis. In conclusion, ZY can be used in treatment of gastric cancer.

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RAPID COMMUNICATION

Clinical and genetic characteristics of Chinese hereditary nonpolyposis colorectal cancer families

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Abstract

AIM: To analyze the clinical characteristics of Chinese hereditary nonpolyposis colorectal cancer (HNPCC) families and to screen the germline mutations of human mismatch repair genes hMLH1 and hMSH2 in the probands.

METHODS: Thirty-one independent Chinese HNPCC families were collected in Zhejiang Province. All of them met Chinese HNPCC criteria. Clinical data about patient gender, site of colorectal cancer, age of onset, history of multiple colorectal cancer, associated extracolonic cancer were recorded. PCR and denaturing high performance liquid chromatography (DHPLC) were employed to screen the mutations. Sequencing analysis was used to find out the exact mutation site and characteristics of the samples showing abnormal DHPLC profiles.

RESULTS: One hundred and thirty-six malignant neoplasms were found in 107 patients including 14 multiple cancers. One hundred and six of the 136 neoplasms (77.9%) were diagnosed as colorectal cancer, with an average age of onset at 48.57 ± 29.00 years. Gastric cancer was the most common extracolonic cancer (10.3%) in these families. Twenty-three different sequence variations in hMLH1 and hMSH2 genes were detected in these 17 families. Fifteen sequence variations were located in the exons, including 5 SNPs, 3 silent mutations, 3 missense mutations, 2 nonsense mutations and 2 frameshift mutations. The latter seven mutations seemed to be pathogenic.

CONCLUSION: Germline mutations of hMLH1 and hMSH2 genes are identified in about one-third HNPCC kindreds fulfilling Chinese HNPCC criteria. Chinese HNPCC families have some particular clinical characteristics, such

as a left-sided predominance, less synchronous or metachronous colorectal cancer, and frequent occurrence of gastric cancer.

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Key words: Colorectal cancer; Hereditary nonpolyposis; DNA mutation analysis; High pressure liquid Chromatography; Oncogenes

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<http://www.wjgnet.com/1007-9327/12/4074.asp>

INTRODUCTION

Hereditary nonpolyposis colorectal cancer (HNPCC) is an autosomal dominantly-inherited cancer susceptibility syndrome. It is estimated that HNPCC may account for 5%-10% of the total colorectal cancers (CRC) worldwide^[1]. Clinically, HNPCC families are diagnosed based on the Amsterdam criteria. Although the Amsterdam criteria unify the diagnosis of HNPCC worldwide, they are too rigid to exclude extracolonic cancers associated with HNPCC. New criteria and guidelines have been proposed, such as the Japanese criteria, suspected HNPCC criteria. Based on the suspected HNPCC criteria and the specific characteristics of the tumor spectrum in Chinese population, the Chinese Hereditary Colorectal Cancer Collaboration has established the criteria for Chinese HNPCC families^[2].

Six genes (hMSH2, hMLH1, hPMS1, hPMS2, hMSH6/GTBP and hMLH3) involved in DNA mismatch repair have been proven to be closely related with the development of HNPCC. hMLH1 and hMSH2 genes are thought to be the main genes responsible for HNPCC, because more than 90% detected germline mutations in HNPCC families are located in these two genes^[3]. Denaturing high-performance liquid chromatography (DHPLC) is a mutation pre-screening method^[4]. The major advantage of DHPLC is the low cost and the high speed of analysis. Therefore, in this study, we analyzed the clinical characteristics of our 31 Chinese HNPCC families registered in our cancer institute, and screened the germline mutations of hMLH1 and hMSH2 genes by DHPLC and DNA sequencing.

MATERIALS AND METHODS

Clinical data

Thirty-one families involved in this study fulfilling the Chinese HNPCC criteria were collected in Zhejiang Province. Detailed familial and medical histories were obtained through an interview with the probands, a home visit to extended family members and an extensive review of medical records if available. Peripheral blood samples were collected from all participants after formal written consent was signed. Eligible HNPCC families were registered and family members were followed up intensively. All patients were reviewed by telephone or by outpatient visit at regular intervals. Data concerning sex, site of CRC, age of diagnosis, history of synchronous and/or metachronous CRC, instance of extracolonic cancers, and histopathology of tumors were documented and thoroughly verified.

Genomic DNA preparation and PCR

Genomic DNA was extracted using the QIAamp DNA isolation kit (Qiagen, Valencia, CA) according to the manufacturer's recommendations. PCR was performed using 100 ng of genomic DNA as template. A 25 μ L reaction mixture containing 10-20 pmol of each primer, 1.5 U of Taq DNA polymerase (Transgenomics, UK) with a final concentration of 2 mmol/L Mg^{2+} and 0.2 mmol/L of dNTPs was used. PCR conditions were as follows: an initial denaturing at 95°C for 5 min, followed by 40 cycles at 95°C for 30 s, at 55-60°C for 30 s, at 72°C for 40 s, and a final extension at 72°C for 10 min. A total of 35 sets of primers including 19 sets for hMLH1 gene and 16 sets for hMSH2 gene were used^[5].

DHPLC analysis

DHPLC analysis was carried out on an automated HPLC device equipped with a DNA separation column (WAVE: Transgenomic, San Jose, CA, USA) as previously described^[6].

DNA sequencing

PCR products displaying abnormal DHPLC peak were purified with microconcentrator filters (Amicon, Beverly, MA) and sequenced with a Bioasia-1524-030/3730DNA sequencer. All mutations were sequenced in both directions.

RESULTS

Clinical characteristics of HNPCC families and probands

A total of 31 kindreds meeting the Chinese HNPCC criteria or the Amsterdam criteria were studied. One hundred and thirty six malignant neoplasms were found in 107 patients (14 multiple cancers), including 106 CRCs, 14 gastric cancers, 3 esophageal cancers, 2 lung cancers, 2 cervical cancers, 2 leukemia, 1 breast cancer, 1 ovarian cancer, 1 oral cancer, 1 thyroid cancer, 1 hepatic cancer, 1 urinary cancer, 1 malignant histiocytosis. CRC accounted for 77.9% (106/136) of the cancers. Nine multiple colorectal tumors and 5 colorectal tumors were associated with extracolonic cancer, accounting for 8.5% and 4.5% of the total CRCs, respectively. Of the 106 CRCs, 18 were located in the

right-sided colon, 8 in the transverse colon, 15 in the left-sided colon, 25 in the rectum and 40 in unidentified site. Left-sided colon cancers constituted of 60.6% (40/66) of all CRCs. Individuals suffering from gastric cancer accounted for 10.3% (14/136) of total patients. The average age of malignant neoplasm onset in all the patients was 48.82 ± 13.32 years and the ratio of males to females was 1.7:1. The average age of CRC patients was 48.57 ± 29.00 years. All predispositions in kindreds were followed up.

Mutations of hMLH1 and hMSH2 genes

A total of 23 abnormal peak profiles were found in 31 probands from 31 families. Finally, 17 probands were identified with 23 base variations by sequencing analysis, of which 7 base substitutions were located in the hMLH1 gene (6 in the exons, 1 in the intron) while the other 16 base substitutions were located in the hMSH2 gene (9 in exons, 7 in introns). Of the fifteen base substitutions in coding sequences, five were proven to be polymorphisms (SNPs), while the other ten were proven to be mutations, including 3 missense mutations (samples H4, H11 and H12), 2 nonsense mutations (samples H20 and H27), 2 frameshift mutations (samples H24 and H29), and 3 silent mutations (samples H5, H16 and H28). The detailed information is shown in Table 1 and Figure 1.

DISCUSSION

HNPCC is characterized by dominant right colon localization, early age of onset, high prevalence of synchronous and metachronous tumors, and certain extracolonic cancers^[14]. In our study, patients with Chinese HNPCC developed CRC at an average age of 48.57 ± 29.00 years. As compared to the onset of sporadic colorectal malignancies at the age approximately 60 years, the onset of HNPCC was much earlier, similar to that in Western countries. In the present study, synchronous and metachronous colorectal cancers only accounted for 8.5% of all CRCs, lower than those in Western countries. The reason is still unclear. Left-sided colorectal cancers accounted for 60.6% (40/66) of all CRCs. This result might be due to the high incidence of left-sided colon cancer (including rectal cancer) in Chinese population. Gastric cancer was the most frequent extracolonic cancer in our 31 families, while endometrial cancer was the most common in Western countries. The reason might be due to the high incidence of gastric cancer in Chinese population. Liu *et al.*^[15] have reported similar results found in their cohort of Chinese HNPCC families. These characteristics might truly reflect the specific clinical phenotype of Chinese HNPCC.

In this study, we screened mutations of the hMLH1 and hMSH2 genes in 31 Chinese HNPCC families by DHPLC and PCR as well as DNA sequencing. Twenty-three sequence abnormalities were detected in 17 probands, 15 in exons and 8 in introns. Base variants identified were compared with those described in the human gene mutation database (HGMD) (<http://www.uwcm.ac.uk/uwcm/mg/hgmd0.html>). Seven of the 15 sequence abnormalities including 3 missense mutations (H4, H11, H12), 2 nonsense mutations (H20, H27), and 2 frameshift mutations (H24, H29) in coding regions of the hMLH1

Table 1 hMSH2/hMLH1 gene sequence variations identified by sequencing in Chinese HNPCC

Family	Gene/exon	point of mutation	Peptide change	Mutation result	Significance	Reported by
H3	hMLH1/12	g.1151T > A,GTT-GAT	Val-Asp, V384D		SNP	Wang Y <i>et al</i> ^[7]
H4	hMLH1/2	g.199G > A,GGG-AGG	Gly-Arg, G67R	missense mutation	pathological	Sasaki S <i>et al</i> ^[8]
H5	hMSH2/1	g.54C > G,GGC-GGG	Gly-Gly ,G18G	silent mutation	synonymous	
H6	hMSH2/7	g.1276 + 47T>A		intronic	uncertain	
H7	hMLH1/8	g.637G > T,GTG-TTG	Val-Leu, V213L		SNP	
H11	hMLH1/15	g.1668-20A > G		intronic	uncertain	
	hMSH2/12	g.1886, A > G,CAA-CGA	Gln-Arg, Q629R	missense mutation	pathological	Kim JC <i>et al</i> ^[9]
H12	hMSH2/15	g.2516, A > G,CAT-CGT	His-Arg, H839R	missense mutation	pathological	
H13	hMSH2/10	g.1661 + 12G > A		intronic	SNP	Scott RJ <i>et al</i> ^[10]
H15	hMSH2/1	g.211+ 9C > G,		intronic	SNP	
H16	hMLH1/8	g.649, C > G,CGC-TGC	Arg-Cys, R217C		SNP	Miyaki M <i>et al</i> ^[11]
	hMSH2/7	g.1221, C > G,CTC-CTG	Leu-Leu, L407L	silent mutation	synonymous	
	hMSH2/13	g.2006-6T > C		intronic	SNP	
H20	hMLH1/19	g.2250, C > G,TAC-TAG	Tyr-X, Y750X	nonsense mutation	truncated peotein	Syngal S <i>et al</i> ^[12]
H21	hMSH2/1	g.23C > T, ACG-ATG	Thr-Met, T8M,		SNP	Nomura S <i>et al</i> ^[13]
	hMSH2/1	g.211 + 9C > G,		intronic	SNP	
H22	hMSH2/1	g.23C > G, ACG-ATG	Thr-Met, T8M		SNP	Nomura S <i>et al</i> ^[13]
	hMSH2/1	g.211 + 9C > G,		intronic	SNP	
H24	hMSH2/11	g.1664, delA,	Stopat odon556	frameshift mutation	truncated protein	
	hMSH2/11	g.1662-2A > G		intronic	uncertain	
H27	hMSH2/11	g.2292G > A, TGG-TGA	Trp-Stop, W764X	nonsense mutation	truncated protein	
H28	hMSH2/5	g.795T > C,GTT-GTC	Val-Val, V265V	silent mutation	synonymous	
H29	hMLH1/14	g.1591delGT	Stop at codon555	frameshift mutation	truncated protein	

g: genomic DNA

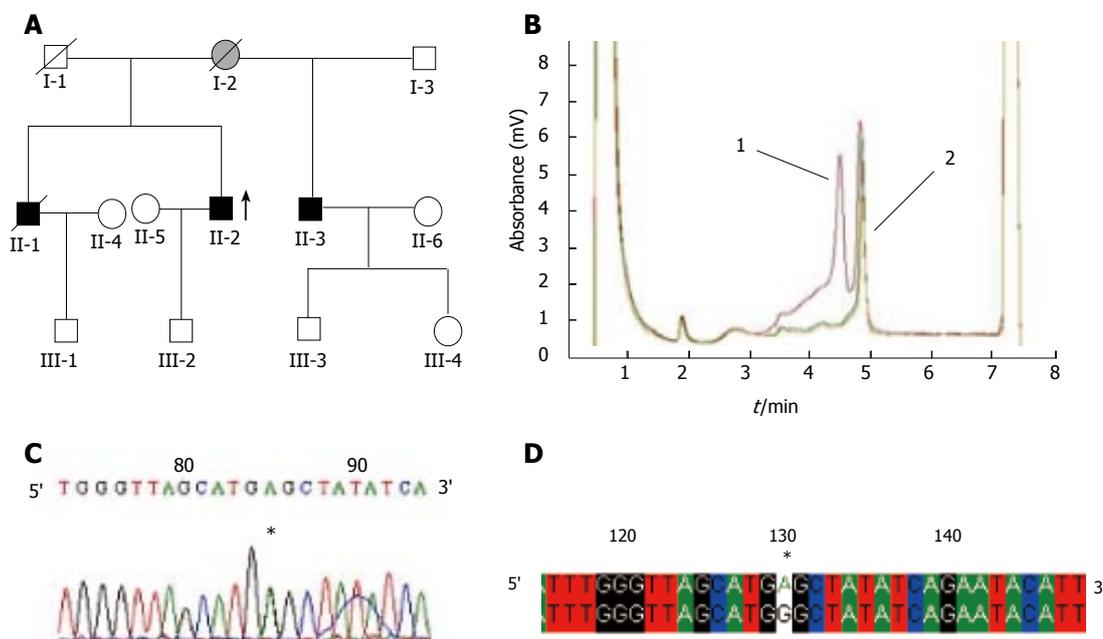


Figure 1 Mutations of hMLH1 and hMSH2 genes. **A:** pedigree map. I-2: cervical cancer at age of 42 years; II-1: rectum cancer at age of 38 years; II-2: proband, transverse colon cancer at age of 51 years; II-3: transverse colon cancer at age of 49 years; **B:** DHPLC analysis. 1: proband's abnormal profile; 2: normal control; **C:** sequencing graphs of mutation. *: the position of mutation G > A; **D:** mutation analysis. upper lane: result of proband's sequencing; lower lane: normal control.

and hMSH2 genes might probably affect the protein functions. Detailed information of these mutations is listed in Table 1. Among them, mutations of H4, H11 and H20 have been reported before^[8,9,12], while the other four mutations of H12, H27, H24 and H29 are novel. The mutation rate was 22.6% (7/31). Nearly half of the mutations were missense mutations. Three (H5, H16, H28) of the fifteen sequence abnormalities in coding sequences are novel silent mutations in exons 1, 7, 5 of the hMSH2 gene, respectively. The last five (H3, H7, H16, H21 and H22) of

the fifteen sequence abnormalities in coding sequences are known as SNPs^[7,10,11,13].

The missense mutation detected in sample H4 located at codon 67 in exon 2 of the hMLH1 gene, has been reported as a pathological mutation^[8]. The missense mutation detected in sample H11 located at codon 629 in exon 12 of the hMSH2 gene has also been reported^[9]. In sample H12, a novel missense mutation at codon 839 in exon 15 of the hMSH2 gene was first discovered in our study, which is located in the Muts V structural motif

(peptides 619-854) of the hMSH2 gene and leads to amino acid change from histidine to arginine. In order to determine the significance of this mutation, 100 blood samples from cancer-free donors were tested under the same conditions and none of the 100 normal samples harbored the same base change as sample H12, suggesting that this base change is pathologic.

The nonsense mutation detected in sample H20 altered nucleotide 2250 (genomic DNA) from C to G at codon 750 in exon 19 of the hMLH1 gene, resulting in a stop codon instead of tyrosine, forming a truncated protein of MutL losing 6 peptides from 751 residue to 756 residue. This nonsense mutation has been reported by Syngal *et al.*^[12]. The other nonsense mutation in sample H27 altered nucleotide 2292 (genomic DNA) from G to A at codon 764 in exon 11 of the hMSH2 gene, leading to the substitution of a stop codon TGA for tryptophan condon TGG, forming a truncated protein of MutC structural motif (peptides 619-854) losing 170 peptides from 765 residue to 934 residue, suggesting that these nonsense mutations may have a causative effect on cancer predisposition and further studies are needed to examine their co-segregation with the phenotype.

Two frameshift mutations detected in samples H24 and H29 have not been reported previously. In sample H24, the base A deleted at codon 555 in exon 11 of the hMSH2 gene led to a stop codon at codon 556. While in sample H29, the GT deleted at codon 531 in exon 14 of the hMLH1 gene led to a stop codon at codon 555. Both mutations produced truncated proteins with insufficient function.

Of the 23 base variants, 8 were located in intronic regions. Five of these 8 variants have been previously reported and known as SNPs (<http://www.ncbi.nlm.nih.gov/>), the significance of the other 3 remains uncertain.

DHPLC is an elegant, new method for mutation detection. It detects heteroduplex formation in renatured PCR fragments possessing heterozygous sequence variations, and has been applied successfully to mutation screening of various genes^[3]. In the past few years, we have searched successfully for mutations in hMLH1 and hMSH2 genes in 29 HNPCC families and 34 suspected HNPCC families with PCR-SSCP and DNA sequencing methods^[16]. However, SSCP is inefficient in detecting mutations and misses 20% of point and frameshift mutations. Though DNA sequencing is supposed to be a screening method with a high sensitivity, it is time consuming and expensive. It was reported that the sensitivity of DHPLC for screening mutations of the hMLH1 and hMSH2 genes can reach 97% without false negatives^[6]. Furthermore, the system is highly automatic, the running time per sample averages only 7 min. Because of these advantages of DHPLC, it enables a broad screening of HNPCC families for human mismatch repair gene alterations in routine analysis.

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RAPID COMMUNICATION

Experimental and clinical study of influence of high-frequency electric surgical knives on healing of abdominal incision

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Abstract

AIM: To study the influence of high-frequency electric surgical knives on healing of abdominal incision.

METHODS: Two hundred and forty white rats were divided into 10^0 , 10^2 , 10^5 , and 10^8 groups and rat models of abdominal operation were induced by using electric surgical knives and common lancets respectively. Then they were respectively given hypodermic injections of normal saline and 0.2 mL quantitative mixture of *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* at a concentration of 10^2 , 10^5 and 10^8 . On the basis of the animal experiment, 220 patients undergoing abdominal operations (above type II) were randomly allocated into one of following three groups: electric knife (EK, 93 cases), electro-coagulation (EC, 55 cases) and control (72 cases). High-frequency electric surgical knives were used to dissect abdominal tissues and electro-coagulation for hemostasis in EK group. Common lancets and electro-coagulation were applied in EC group. Common lancets and tying silk suture were used in the controls.

RESULTS: In all the groups except group 10^0 , infection rate of incisional wounds made by electric surgical knives were remarkably higher than that with common lancets. Furthermore, there were significant differences in groups 10^2 , 10^5 , and 10^8 ($P < 0.05$), but not in group 10^0 ($P > 0.05$) between EK and EC groups. Clinical studies showed a delayed wound healing in 16 cases (17.20%) in EK, 11 cases (16.36%) in EC and 2 cases (2.86%)

in the control groups. A significant difference between EK and the control groups ($\chi^2 = 8.57$, $P < 0.01$), and between EC and the control groups ($\chi^2 = 5.66$, $P < 0.05$) was observed, but not between EK and EC ($\chi^2 = 0.017$, $P > 0.05$).

CONCLUSION: High-frequency electric knives may remarkably delay abdominal incision healing. Its application should be minimized so as to reduce the possibility of postoperative complications.

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Key words: High-frequency electric surgical knives; Abdominal incision; Healing; Infection

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<http://www.wjgnet.com/1007-9327/12/4082.asp>

INTRODUCTION

The high-frequency electric surgical knife is one of the common instruments in surgical operations. It has many advantages such as easy operation and good hemostatic effect, *etc.* In our clinical practice we found, however, the poor healing rates as well as high infection rate of incision made by electric knife (EK) were remarkably increasing. In order to approach the influence of high-frequency electric surgical knives on healing of the abdominal incisions, we carried out animal experiments with white rats from May to Dec 1999, and also conducted clinical trials on 220 patients undergoing abdominal operations (above level II) from Dec 1999 to Sept 2001. Our study shows that high-frequency electric knives may remarkably delay abdominal incision healing.

MATERIALS AND METHODS

Animal grouping

Experimental animals of Wistar white rats, weighing 150-180 g, were divided into experimental group (EG)

and control group (CG) at random, 30 each. Every group was subdivided into 10^2 , 10^5 , 10^8 groups according to the number of vaccinated bacteria, and 10^0 group using normal saline instead of vaccinated bacteria.

Preparation of bacteria solution

Standard strain sources: Based on WHO recommendation, frozen, dry and pure *Escherichia coli* ATCC (25 922), *Staphylococcus aureus* ATCC (25 923) and *Pseudomonas aeruginosa* ATCC (27 853) were supplied by the Clinical Trial Center of Ministry of Health.

Bacteria suspension solution preparation: The standard strains were vaccinated into plating culture of goat's blood, cultured at 35°C for 24 h to obtain the bacteria suspension solution with a concentration of 0.5 U (equal to 10^8 cfu/mL), then the bacteria solutions were made into 10^5 cfu/mL and 10^2 cfu/mL bacteria suspension solution for stock use.

Quantitative culture of bacteria suspension solutions: Ten microliters of the 10^2 cfu/mL bacteria solution were subjected to culture by pour plate method. The results showed that *Escherichia coli* was 130 cfu/mL, *Staphylococcus aureus* was 125 cfu/mL, and *Pseudomonas aeruginosa* was 110 cfu/mL. The 3 kinds of standard bacteria strain suspension solutions were mixed at equal amount, the colonies were counted, and its bacteria content was 12.67 cfu/mL. Thus bacteria content of 10^2 group was 24.33 cfu/0.2 mL (actual bacteria content was about 0.24×10^2).

One microliter of 10^5 cfu/mL bacteria solution was taken for pour culture. The results showed that *Escherichia coli* was 14 500 cfu/mL, *Staphylococcus aureus* was 115 000 cfu/mL, and *Pseudomonas aeruginosa* was 95 000 cfu/mL. The 3 kinds of standard bacteria strains were mixed at equal quantity, and the bacteria content was 224 500 cfu/mL. Therefore, bacteria content of 10^5 group was 44 900 cfu/0.2 mL (actual bacteria content was about 4.49×10^4).

The 10^8 cfu/mL bacteria solution was diluted to 1/50 of its concentration, and 1 μ L of it was taken for pour culture, the colonies were counted after 24 hours. The results showed that *Escherichia coli* was 130 000 000 cfu/mL, *Staphylococcus aureus* 125 000 000 cfu/mL, and *Pseudomonas aeruginosa* 115 000 000 cfu/mL. The 3 kinds of standard bacteria strains were mixed at equal quantity, and the bacteria content was 123 333 333 cfu/mL. Therefore, bacteria content of 10^8 group was 41 111 111 cfu/0.2 mL (actual bacteria content was about 4.11×10^7).

Experimental procedures

After the animals were anesthetized by ether, the experimental groups were operated with EK through midline incision of abdomen, while the control group with common surgical knives. The No 4 silk thread was used to suture peritoneum. According to the group division, 0.2 mL of the mixed bacteria solution was injected subcutaneously. The skin was sutured, and animals were marked and raised in different cages.

Observational parameters

Criteria for incisional healing: According to Surgery^[1]. The healing is classified as A: first-grade healing, B: second-grade healing, and C: third-grade healing.

Table 1 Comparison of operative incision of 3 groups

Group	n	Type I incision n (%)	Type II incision n (%)	Type III incision n (%)
EK	93	6 (6.45)	83 (89.23)	4 (4.30)
EC	55	3 (5.45)	50 (90.91)	2 (3.64)
Control	72	5 (6.94)	63 (87.50)	4 (5.56)

Grading of incisional infection: Non-infection (Grade I): presence of some granulation tissues in the section. Slight infection (Grade II): presence of a few neutrophil diffuse infiltration, the inflammatory area is smaller than 10% of the section. Moderate infection (Grade III): presence of much neutrophil infiltration, the inflammatory area is between 10% to 30% of the section. Severe infection (Grade IV): presence of severe neutrophil infiltration, the inflammatory area is larger than 30%.

Criterion for grease liquefaction: All those that have bad incision healings and meet the following descriptions could be diagnosed as grease liquefaction: (a) Incision has serum-like solution on the surface of incision. (b) Fat drops are observed with naked eyes or microscopy. (c) No purulent secretion is found. (d) Redness and swelling around the incision. (e) No growth in bacteria cultural medium.

Clinical studies

Patient grouping: A total of 220 patients were randomly divided into EK group, EC (electric coagulation) group, and common surgical knives group (control group). EK group consisted of 93 cases, including 57 males, 36 females, with age ranging from 21 to 80 (average 53.70) years. Fifty-seven underwent biliary system operation, 17 colorectal operation, 8 gastric operation, 5 portal hypertension cutout operation, 3 pancreatic operation, 3 respectively splenectomy, resection of small intestine and intestinal tuberculosis operation. EC group consisted of 55 cases, including 30 males, 25 females, with age ranging from 28 to 67 (average 51.54) years. The patients underwent biliary system operation (including one case for subtotal gastrectomy) (30 cases), gastric operation (11 cases), colorectal operation (4 cases), appendicitis exploratory laparotomy (3 cases), small intestine operation and portal hypertension cutout operation (2 cases each), enterolysis, pancreatic operation and liver operation (1 case each). Control group consisted of 72 cases (38 males, 34 females), with age ranging from 22 to 78 (average 55.09) years. Patients underwent biliary system operation (40 cases), gastric operation (10 cases), colorectal operation (7 cases), small intestine operation (5 cases), portal hypertension cutout operation and appendicitis exploratory laparotomy (3 cases each), abdominal mass operation (2 cases), hepatorrhesis with hepatorrhaphy and splenectomy (1 case each).

There were no remarkable differences in sex, age, and types of operative incision among all 3 groups. Table 1 shows the comparison of types of operative incision.

Surgical operation methods: The ways of preoperative preparation and operative disinfection and dressing in the 3 groups were the same. Operative incisions were mostly transrectus incisions, with a few other incisions. EK group

Table 2 Infection of incisional sites for the experimental animals

Group	n	EG's incision healing			Infection rate (%)	n	CG's incision healing			Infection rate (%)
		A	B	C			A	B	C	
10 ⁰	30	23	2	5	23.33	30	28	0	2	6.60
10 ²	27	17	3	7	37.04	30	26	1	3	13.33
10 ⁵	30	15	5	10	50.00	29	22	3	4	24.14
10 ⁸	30	11	4	15	63.33	30	19	2	9	36.67

used EK to resect tissues, and EC for hemostasis. EC group used common surgical knives to dissect tissues, and EC for hemostasis. Control group used common surgical knives, and silk thread suturing for hemostasis. Antibiotics were routinely used, and dressings were changed to observe the healing state of the incisions on the 3rd and 7th d after operation.

Instruments

EK group and EC group used GD350-D high-frequency electric surgical knives, made by Shanghai Hutong Electric Instrument Factory. Power of electric current was set at 40 W. Control group used common surgical knives.

Statistical analysis

Statistical analysis used chi-square test (χ^2). $P < 0.05$ was taken as significant.

RESULTS

Results of the animal experiments

Four white rats died of anesthesia accidents. The other 236 rats were put to death by cut-cord operation. On the 8th post-operative day, infection of incision sites was recorded, and then the 1 cm tissues around the original incision site were fusiformly excised for pathological examinations.

The comparison of incisional sites infection due to different density bacteria between the EG and CG are shown in Table 2.

Statistically significant differences in 10², 10⁵, and 10⁸ groups ($\chi^2 = 4.31, 4.22, 4.27$, respectively $P < 0.05$), but not in Group 10⁰ ($\chi^2 = 3.28, P > 0.05$) were observed.

The comparison of pathological infection of incision due to different density bacteria is shown in Table 3.

Statistically significant differences in groups 10², 10⁵ and 10⁸ ($\chi^2 = 4.05, 4.23, 5.08$, respectively $P < 0.05$), but not in group 10⁰ ($\chi^2 = 0.27, P > 0.05$) were observed.

All the above results showed there were differences in the infection rate based on either experimental observation or pathological examinations. However, both of them indicated that the bacterial contamination would increase the operative infection rate of EK, while the aseptic operation showed no significant difference.

Clinical study results

Second grade or 3rd grade healing (cacoethic healing) accounted for 16 cases (17.20%) in EK; 11 cases (16.36%) in EC, and 2 cases (2.86%) in the control. A significant difference between EK and the control was observed ($\chi^2 = 8.57, P < 0.05$). Furthermore, significant differences

Table 3 Pathological examination results of experimental animals

Group	n	EG				Infection rate(%)	n	CG				Infection rate(%)
		I	II	III	IV			I	II	III	IV	
10 ⁰	30	14	4	9	3	23.33	30	16	7	4	3	6.60
10 ²	27	5	5	10	7	37.04	30	13	5	5	7	13.33
10 ⁵	30	6	7	6	11	80.00	29	13	3	6	7	55.17
10 ⁸	30	5	5	8	12	83.33	30	13	3	5	9	56.67

were found between EC and the control ($\chi^2 = 5.66, P < 0.05$), but not between EK and EC groups ($\chi^2 = 0.017, P > 0.05$).

DISCUSSION

Common reasons for postoperative incisional site infection in laparotomy

Postoperative incisional site infection is a common complication in laparotomy. There are various reasons for infections, for example, without tight hemostasis on operative incisions, incisional contamination, and less strict aseptic operation. In spite of use of large doses of broad-spectrum antibiotics, this problem still has not been satisfactorily solved.

In 1917s, a French doctor Doyen invented EK^[2]. Since then, surgeons have been in favor of wide use of EK, merely because EK can make hemostasis quick and satisfactory, save the operative time and decrease bleeding during operation. In recent years, EK is more and more widely used in surgical operations. Because of unlimited and incorrect use of it, the injury of tissues is worsened and postoperative complications are increasing. Our experiment also testified this. In addition to incision site infection, there are also some grease liquefaction^[3] and abdominal incision deliscence, which are called delayed incision healing.

Early in the 1970s, Link *et al*^[2] proposed that EK could delay incision healing. The histological reports showed that the injury of electrocauterized tissue was worsened and the incisional site infection rate was increased. Studies by Soballe^[4] and Kumagai^[5] also indicated that EK could decrease the threshold of the infection in abdominal incisions.

Our animal experiments demonstrated that in each subgroup, the infection rate of incisional wounds made with EK was remarkably higher than that in the control group. There were significant differences in groups 10², 10⁵ and 10⁸ ($\chi^2 = 4.31, 4.22, 4.27$, respectively $P < 0.05$), except for group 10⁰ ($\chi^2 = 3.28, P > 0.05$). Since experimental pathological examination results were the same as experimental observational results, it indicated that EK could increase the infection rate of non-sterilized incisions, but not increase the infection rate of aseptic incisions.

Although clinical data were controversial on this issue, our current study demonstrated that there was a significant difference between EK and the control ($\chi^2 = 8.57, P < 0.01$). In addition, significant differences were observed between EC and the control ($\chi^2 = 5.66, P < 0.05$), but not between EK and EC ($\chi^2 = 0.017, P > 0.05$). It was proved

that EK may delay abdominal incision healing, while EC may not. Therefore, we suggest avoiding use of EK, and recommend use of common surgical knives when opening abdomen, and tying or EC for hemostasis.

Reasons for increased rate of incision cacoethic healing by EK

Local hyperthermy: When the temperature is higher than 45°C to 50°C, it can cause tissues and cells to denature; when the temperature is higher than 60°C, it can cause tissue necrosis. As the local temperature caused by EK can reach 200°C to 1000°C, if tissue is burnt under a big power again and again, a large number of histiocytes can be destroyed, which can cause incision wound hydrops and cacoethic healing.

Local tissue ischemia: When EK is being used, electric current and thermal energy are dispersed radiatively along the incisive margin, thereby causing denaturation and vascular occlusion in tissues on both sides of the incisive margins, and also the disturbances of local blood supply, thus affecting incisional healing. How *et al*^[2] have proved that tissues without blood vessels are more likely to be infected than those that have blood vessels.

Condition favorable for bacteria growth: The high temperature of EK can destroy the histiocytes, and lipocytes are more sensitive to thermal energy. A great amount of lipocytes destruction which can cause grease efflux, and coagulation tissues are good culture media for bacteria. Bacterial infection can cause incisional infection. That is why EK may increase the infection rate of non-sterilized incisions while has little effect on aseptic incisions.

Local hyperosmotic state: When the lipocytes are destroyed, micromolecular material makes the incisions in the hyperosmotic state, which accelerates subcutaneous hydrops, leading to incisions cacoethic healing.

Suggestions on application of EK

In our view, we should strictly follow the indications for use of EK. For complicated operations or operations of serious patients, such as reoperated patients, malignant tumor, in order to avoid excessive blood loss of patients,

save operative time, we should limitedly use EK. In another word, when opening abdomen, EC should be preferable to electrotony and the electric current should be under control while using EC for hemostasis. When using electrotony and EC in abdominal cavity operation, we also should control electric current to decrease tissue destruction, and the incision cacoethic healing rate. However, Kuzon^[6] failed to find any significant difference in the rate of the incisional complication between the high electric current volume and low electric current volume. Another point to be mentioned is, use of gauze before the operation is finished also has some positive effect on preventing incision liquefaction by getting rid of lipid and adipose tissues.

There are various causes for the infection of abdominal incision. Therefore, we should strictly follow the aseptic operating principle in the operation, protect incisions, avoid bacteria contamination, and make tight suturing for hemostasis. Meanwhile, prophylactic use of antibiotics in preoperative and postoperative periods is an effective way to decrease the infection rate of incisions.

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S- Editor Wang J L- Editor Zhu LH E- Editor Liu WF

RAPID COMMUNICATION

***In vitro* screening of traditionally used medicinal plants in China against Enteroviruses**

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Abstract

AIM: To search for new antiviral agents from traditional Chinese medicine, specifically anti-enteroviruses agents.

METHODS: The aqueous extracts (AE) of more than 100 traditionally used medicinal plants in China were evaluated for their *in vitro* anti-Coxsackie virus B3 activities with a MTT-based colorimetric assay.

RESULTS: The test for AE of 16 plants exhibited anti-Coxsackie virus B3 activities at different magnitudes of potency. They can inhibit three steps (inactivation, adsorption and replication) during the infection. Among the 16 plants, *Sargentodoxa cuneata* (Oliv.) Rehd. et Wils., *Sophora tonkinensis* Gapnep., *Paeonia veitchii* Lynch, *Spatholobus suberectus* Dunn. and *Cyrtomium fortunei* J. sm. also have activity against other enterovirus, including Coxsackie virus B5, Polio virus I, Echo virus 9 and Echo virus 29. Cell cytotoxic assay demonstrated that all tested AE had CC₅₀ values higher than their EC₅₀ values.

CONCLUSION: The sixteen traditionally used medicinal plants in China possessed antiviral activity, and some of them merit further investigations.

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Key words: Traditional used medicinal plant; China; Antiviral activity; Enterovirus; *Sargentodoxa cuneata* (Oliv.) Rehd. et. Wils.; *Sophora tonkinensis* Gapnep.; *Paeonia veitchii* Lynch.; *Cyrtomium fortunei* J. sm.; *Spatholobus suberectus* Dunn

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INTRODUCTION

Human enteroviruses, the largest genus of the *Picornaviridae* family, are of great medical and economic importance causing a variety of clinical syndromes and diseases. Enterovirus can cause any of the syndromes and, vice versa, any syndrome or disease could be the result of infection by the enterovirus. Coxsackieviruses, part of the enterovirus genus, can cause severe diseases of the heart, liver, eyes and pancreas, as well as acute infections of the central nervous system. Nowadays, Coxsackieviruses B are the major etiological agents of human myocarditis, causing between 25% and 35% of cases for which a cause is found^[1]. Transition from acute myocarditis to dilated cardiomyopathy has been suspected^[2]. So far, dilated cardiomyopathy is one of the major reasons for cardiac transplantation. Coxsackieviral RNA in the myocardium can be a marker of a poor clinical outcome and might influence prognosis after heart transplantation^[3]. Enteroviruses and Coxsackieviruses in particular, have been implicated in several chronic illnesses including juvenile onset diabetes mellitus, chronic fatigue syndrome, dermatomyositis and polymyositis, congenital hydrocephalus and amyotrophic lateral sclerosis^[4-6]. Until recently, there were no enterovirus-specific drugs available for clinical use^[7]. A great number of picornavirus replication inhibitors *in vitro* have been described but just few of them have shown effectiveness *in vivo*^[8], and none has been approved for clinical use yet. Etiological therapy for enteroviral diseases still remains elusive. The main reason for that is the fast development of drug-resistant and even drug-dependent mutants^[9,10]. Use of synergistic combinations of antivirals might be one of the possible efficient approaches to overcome the disadvantages of monotherapy. The same or greater effect could be achieved at lower concentrations than those required if drugs were to be used alone. Combined chemotherapy may also restrict the emergence of resistance to either or both of the partners in the combination. Also, research on the antiviral effect of combinations of picornavirus inhibitors might contribute to the better understanding of their mode of action and, in general, the mechanism of picornavirus replication. Currently, there is no specific antiviral therapy to treat or prevent enterovirus disease. Thus, new and more effective antiviral agents for future therapy in enterovirus infection are desired.

The development of a new antiviral drug is a difficult task taking into account the poor selective toxicity and fast selection of resistant viral variants with the existing

drugs. Frequencies of viral resistance to antiviral drugs are increasing and the difficulty of virus latency remains unsolved.

The screening of plants as a possible source of antiviral agents has led to the discovery of potent inhibitors of *in vitro* viral growth^[11-17] and the use of the ethnopharmacological approach enhances the probability of identifying new bioactive plant compounds^[18,19].

Plants have long been used as remedies against infection diseases. Many plant preparations have been used externally as disinfectants and antiseptics for wounds and pimples, as antidiarrhoeics and in the treatment of respiratory diseases. Nowadays, these are still used by rural populations. Plants considered useful against infectious diseases are interesting to test with regard to different etiological agents (viral, bacterial, fungus).

In our continuous efforts to search for novel antiviral agents from traditional medicinal plants, more than one hundred traditionally used medicinal plants in China were extracted with water and investigated for their *in vitro* anti-enterovirus activities. This is the first report on screening traditionally used medical plants against enterovirus.

MATERIALS AND METHODS

Preparation of the extracts

All traditionally used medicinal plants were purchased from Darentang Pharmaceutical Co. Ltd, a famous local traditional Chinese medicine provider in Tianjin city of China. All plant materials were air dried, ground and powdered. Hot water extracts of the traditionally used medicinal plants were prepared according to the procedures as described below: different medicinal plant materials (20 g) were boiled with 1000 mL of distilled water for 2 h. The aqueous was collected and the residual was extracted again with another 1000 mL of distilled water. The resulting aqueous extracts were collected, combined, filtered by gauze, concentrated under reduced pressure and lyophilized to dry. The AE extracts were dissolved in sterile distilled water.

Cells and virus

Vero E6 was used as target cells for virus infection. Cells were cultivated using RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin G, 100 µg/mL streptomycin. In the antiviral assay, the medium was supplemented with 2% FBS and the above mentioned antibiotics. All cell culture reagents and media were purchased from Gibco BRL (Grand Island, New York).

Polio virus I was obtained from the American Type Culture Collection (ATCC), Rockville, USA. Coxsackie virus B3 and Coxsackie virus B5 were provided by Professor X.M. Li (Medical University of Tianjin, Tianjin, China). Echo virus 9 and Echo virus 29 were provided by the Chinese Type Culture Collection (CTCC), Wuhan, China. All virus strains were kept in our laboratory. All viruses were prepared and quantitated on Vero E6 cells and stored in small aliquots at -70°C until use.

Titration of viruses

Vero E6 cells were seeded in 96-wells culture plates at a

density of 10⁴ cells/well and then incubated at 37°C in a humidified atmosphere containing 5 mL/L CO₂ for 24 h. A serial dilutions of virus stocks were prepared and cells were infected with the dilution of virus. After an additional 72 h of incubation, the cytopathic effect was recorded. The 50% tissue culture infective dose (TCID₅₀) per mL was calculated as described previously by Reed and Muench^[20].

Antiviral assay using MTT method

The antiviral activity of AE extracts was evaluated by the MTT method based in the color change which occurred following the reduction of 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) by mitochondrial enzymes^[21]. Vero E6 cells, treated by trypsin, were seeded in 96-well culture plates with a volume of 200 µL/well and a concentration of 10⁵ cells/mL. After 24 h incubation, 100 µL of 100 TCID₅₀ of Coxsackie virus B3 was added, and the infected cells were incubated for another 2 h. Tested compound (200 µL) at different concentrations was then added to culture wells in triplicate. After further incubation at 37°C with 5 mL/L CO₂ for 72 h, MTT was added. The culture was incubated for four hours to allow the production of formazan and the absorbance at 492 nm was measured using a 96-well plate ELISA reader (Multiskan EX, Labsystems).

Viral inhibition rate was calculated as follows: [(OD_{tv}-OD_{cv})/(OD_{cd}-OD_{cv})] × 100%.

OD_{tv}, OD_{cv} and OD_{cd} indicate the absorbance of the test compounds with virus infected cells, the absorbance of the virus control and the absorbance of the cell control, respectively. The 50% effectiveness concentration (EC₅₀) was defined as the concentration that achieved 50% cytoprotection against virus infection.

To confirm the results obtained with the MTT assay, the monolayers were also observed microscopically for estimating CPE (i.e. rounding and other marked morphologic changes with respect to control cells).

In addition to the post-incubation method of virus inhibition assay, pre-treatment (adding 2 h before virus infection) and co-treatment (adding at the same time of virus infection) of AE extracts were also attempted in the same protocol as mentioned above except the variation in the time of addition of AE extracts.

Cell cytotoxic effect

The cell cytotoxic effect of tested compounds toward Vero E6 cells was evaluated by MTT-based method. It was performed according to the procedures as described above with no virus added. Cell cytotoxic effect of each tested compound was calculated by the following formula: Percent of cell cytotoxic effect = [1- (OD_t/OD_s)] × 100%.

OD_t and OD_s indicate the absorbance of the test substances and the solvent control, respectively. The 50% cell cytotoxic concentration (CC₅₀) of tested compounds was calculated according to Chiang *et al.*^[22,23].

Statistical analysis

Data were calculated for three separate experiments. The selectivity index (SI) was calculated as the ratio of CC₅₀ to EC₅₀. The Student's unpaired *t*-test was used to calculate

Table 1 *In vitro* anti-Coxsackie virus B3 activity of 16 traditionally used medicinal plants in China

Infusion	Used part	Antiviral activity		
		Viral inactivation	Inhibition of adsorption	Inhibition of replication
<i>Arctium lappa</i> L.	Seeds	+++	+	+
<i>Belam anda chinensis</i> (L.) DC	Stem and root	+++	+	+
<i>Cyrtomium fortunei</i> J. sm.	Stem	++++	+++	+++
<i>Dictamnus dasycarpus</i> Turcz.	Root	+++	+	+
<i>Ephedra Sinica stapf</i>	Stem	++++	+	++
<i>Epimedium brevicornum</i> Maxim.	Stem and leaf	+++	+	+
<i>Herba patriniae</i> .	Whole grass	+++	+	+
<i>Lindera aggregata</i> (Sims) Kostem.	Root	+++	+	+
<i>Lygodium japonicum</i> (Thunb) Sw.	Seeds	+++	+	+
<i>Paeonia lactiflora</i> Pall.	Root	+++	+	++
<i>Paeonia veitchii</i> Lynch	Root	++++	+	++
<i>Plantago asiatica</i> L.	Seeds	+++	+	+
<i>Sargentodoxa cuneata</i> (Oliv) Rehd.et.Wils	Stem	++++	+++	+++
<i>Sophora tonkinensis</i> Gapnep.	Root	++++	+	++
<i>Spatholobus suberectus</i> Dunn	Stem	++++	+++	+++
<i>Terminalia chebula</i> Retz	Seeds	+++	+	+

++++: the strongest antiviral activity; +++: strong antiviral activity; ++: the modest antiviral activity; +: the weak antiviral activity; -: no antiviral activity.

Table 2 EC₅₀ and selectivity index of five traditionally used medicinal plants in China

Infusion	CC ₅₀ (mg/L)	Viruses									
		Coxsackie virus B3		Coxsackie virus B5		Echo virus 9		Echo virus 29		Polio virus I	
		EC ₅₀ (mg/L)	SI								
<i>Guanidine Sargentodoxa</i>	> 1000.0	35.4	28.2	36.7	27.3	18.6	53.8	33.2	30.1	67.8	14.7
<i>Cuneata</i> (Oliv.) Rehd.et Wils	> 500.0	51.2	9.8	29.1	17.2	12.8	39.1	28.2	17.7	43.2	11.6
<i>Sophora tonkinensis</i> Gapnep.	> 125.0	19.2	6.5	77.5	1.6	6.3	19.8	23.2	5.4	14.9	8.4
<i>Paeonia veitchii</i> Lynch.	> 500.0	115.3	4.3	86.2	5.8	43.5	11.5	65.3	7.7	136.8	3.7
<i>Spatholobus suberectus</i> Dunn.	> 250.0	60.8	4.1	47.1	5.3	14.8	16.9	65.5	3.8	29.1	8.6
<i>Cyrtomium fortunei</i> J. sm.	> 250.0	64.9	3.9	66.2	3.8	13.6	18.4	44.2	5.7	52.8	4.7

P values of difference of means between control and the tested samples on the inhibition of virus replication. Difference of sample between tested viruses with a *P* value less than 0.05 was considered statistically significant.

RESULTS

Anti-Coxsackie virus B3 activity of traditionally used medicinal plants in China

Aqueous extracts from 151 traditionally used medicinal plants were studied to detect the activities against Coxsackie virus B3, including effect on viral replication; effect on viral adsorption and subsequent replication and *in vitro* viral inactivation. The results showed that aqueous extracts of 16 used medicinal plants in China exhibited *in vitro* anti-Coxsackie virus B3 during the three different antiviral assays (Table 1). The antiviral activity against Coxsackie virus B3 by estimating cytopathic effect. Among the 16 medicinal plants, all exhibited strong anti-Coxsackie virus B3 activity on *in vitro* viral inactivation. But the extracts from *Sargentodoxa Cuneata* (Oliv.) Rehd.et Wils., *Sophora tonkinensis* Gapnep., *Paeonia veitchii* Lynch., *Paeonia lactiflora* Pall., *Ephedra Sinica stapf*, *Spatholobus suberectus* Dunn. and *Cyrtomium fortunei* J. sm. appeared to possess the strongest anti-Coxsackie virus B3 activity on viral replication; and the extract from *Cyrtomium fortunei* J. sm., *Sargentodoxa Cuneata* (Oliv.) Rehd.et Wils., and *Spatholobus*

suberectus Dunn. can strongly inhibited the adsorption of Coxsackie virus B3 to the cell.

EC₅₀ and selectivity index of five traditionally used medicinal plants against other enterovirus

Table 2 shows EC₅₀ of AE extract of five medicinal plants. Overall, all AE extract showed CC₅₀ higher than their EC₅₀. These observations indicated that the antiviral activity of AE extracts was not a result of their cytotoxic effect toward cells. The CC₅₀ values ranged from 125 to 500 mg/L.

With the EC₅₀ and CC₅₀ data, the selectivity index (SI) was calculated by dividing CC₅₀ by EC₅₀. The SI for the anti-Coxsackie virus B3 assay ranged from 3.9 to 9.8, and 1.6 to 17.2 for the anti-Coxsackie virus B5 assay. For anti-Echo virus 9, Echo virus 29 and Polio virus I assays, the SI ranged from 11.5 to 39.1, 3.8 to 17.7 and 3.7 to 11.6, respectively.

DISCUSSION

Medicinal plants have been traditionally used for different kinds of ailments including infectious diseases. Some of them are reported to exhibited antiviral activity in literature^[15,18]. According to Cragg's report, approximately 60% of anti-tumor and anti-infective agents that are commercially available or in the late stages of clinical trials today are of natural product origin^[24]. There is therefore,

no doubt that traditional medicinal plants can serve as a potential resource in the development of new antiviral agents in the future. Since current chemotherapy agents for enterovirus infections are either insufficient in quantity or limited in efficiency, there is thus a need to search for new and more effective antiviral agents for future therapy in enterovirus infections.

Among the 151 tested medicinal plants, five of them were found to exhibited a broad spectrum of anti-enterovirus activity. These five medicinal plants were *Sargentodoxa cuneata* (Oliv.) Rehd. et Wils, *Sophora tonkinensis* Gapnep., *Spatholobus suberectus* Dunn., *Paeonia veitchii* Lynch. and *Cyrtomium fortunei* J. sm. In China, they were not used to treat the virus infection. *Sargentodoxa Cuneata* (Oliv.) Rehd. et Wils is the dried vine stem of *Sargentodoxa cuneata* (Oliv.) Rehd. et Wils. (Fam. Lardizabalaceae), traditionally used to remove toxic heat, to promote blood circulation, and to relieve rheumatic conditions. *Sophora tonkinensis* Gapnep. is the dried root and rhizome of *Sophora tonkinensis* Gapnep. (Fam. Leguminosae), used to remove toxic heat, promote the subsidence of swelling, and soothe the sore throat. *Spatholobus suberectus* Dunn. is the dried stem of *Spatholobus suberectus* Dunn (Fam. Leguminosae), used to enrich the blood, to activate blood circulation, and to remove obstruction of the channels and collaterals. *Paeonia veitchii* Lynch. is the dried root of *paeonia lactiflora* Pall. or *Peaonia veitchii* Lynch (Fam. Ranunculaceae), used to remove heat from blood, to eliminate blood stasis, and to relieve pain. *Dryopteris crassirhizoma* Nakai is the dried rhizome of *Dryopteris crassirhizoma* Nakai (Fam. Aspidiaceae), used to remove toxic heat, to expel parasites, and to stop bleeding. Our studies revealed that the five medicinal plants suppressed five enteroviruses at different magnitudes of potency. Among them, the aqueous extracts of *Sargentodoxa cuneata* (Oliv.) Rehd. et Wils showed the strongest antiviral activity to all five viruses and noteworthy SI value (SI > 4.0). The SI of five medicinal plants to five viruses values from 1.6 to 39.1 suggested a promising future for these extracts as antiviral products. It is important to emphasize there has been no a novel drug for enteroviruses diseases.

Anti-enteroviruses of aqueous extracts of these five plants against five enteroviruses *in vitro* have been demonstrated. It is not known, however, what these results signify for *in vivo* effectiveness. The results obtained in this preliminary screening justify continuing with the purification of crude extracts and isolation of active compounds for improving their potential as antiviral drugs and/or finding of new lead molecules.

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RAPID COMMUNICATION

Whole-cell recordings of calcium and potassium currents in acutely isolated smooth muscle cells

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Abstract

AIM: To record calcium and potassium currents in acutely isolated smooth muscle cells of mesenteric arterial branches in rats.

METHODS: Smooth muscle cells were freshly isolated by collagenase digest and mechanical trituration with polished pipettes. Patch clamp technique in whole-cell mode was employed to record calcium and potassium currents.

RESULTS: The procedure dissociated smooth muscle cells without impairing the electrophysiological characteristics of the cells. The voltage-gated Ca^{2+} and potassium currents were successfully recorded using whole-cell patch clamp configuration.

CONCLUSION: The method dissociates smooth muscle cells from rat mesenteric arterial branches. Voltage-gated channel currents can be recorded in this preparation.

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Key words: Patch clamp; Smooth muscle cell; Voltage-gated channel; Whole-cell recording

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INTRODUCTION

Ca^{2+} and K^{+} channels, two components that form action potential, are especially important for the regulation of cell excitability because they depolarize and repolarize excit-

able cells in response to depolarizing events. Voltage-gated K^{+} channels are implicated in governing cell excitability^[1] and setting resting potential^[2]. Studies have shown that molecular mechanisms, including protein phosphorylation, gene expression, muscle contraction and neurotransmitter release, are regulated by Ca^{2+} channels^[3]. Patch clamp technique is a powerful tool for studying the electrophysiological properties of biological membranes. Although some methods for patch clamp using freshly isolated smooth muscle cells have been described^[4], these methods need multiple enzymes^[5] or complex procedure^[6]. An improved method was described in the present study for the fast and reliable isolation of smooth muscle cells of mesenteric arterial branches by single collagenase digest and mechanical trituration. The present study was to establish the method of whole-cell recordings of calcium and potassium currents in acutely isolated smooth muscle cells. It can be used to observe the effects of drugs and some gastrointestinal tract disease on ion channels of smooth muscle cells of gastrointestinal tract.

MATERIALS AND METHODS

Solution and drugs

The Krebs-Ringer solution containing 120.7 mmol/L NaCl, 5.9 mmol/L KCl, 15.5 mmol/L $NaHCO_3$, 1.2 mmol/L NaH_2PO_4 , 1.2 mmol/L $MgCl_2$, 2.5 mmol/L $CaCl_2$ and 11.5 mmol/L glucose was used in this study with its pH adjusted to 7.4. The Ca^{2+} -free physiological saline solution (PSS) containing 150 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L $MgCl_2$, 10 mmol/L HEPES, and 10 mmol/L glucose was also used in the study. The pH was adjusted to 7.4 with Tris-base. For recording Ca^{2+} currents, the cells were continuously perfused with the extracellular solution containing 140 mmol/L Teal, 5 mmol/L $BaCl_2$, 10 mmol/L HEPES, 10 mmol/L glucose. The pH was adjusted to 7.4 with Tris-base. Recording pipettes were filled with the following intracellular solution containing 130 mmol/L CsCl, 10 mmol/L EGTA, 3 mmol/L MgATP, 0.3 mmol/L Na_2GTP , 10 mmol/L HEPES, 10 mmol/L glucose. The pH was adjusted to 7.2 with CsOH. For recording potassium currents, the cells were continuously perfused with HEPES-buffered physiological containing 150 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L $MgCl_2$, 2 mmol/L $CaCl_2$, 10 mmol/L HEPES, 10 mmol/L glucose. The pH was adjusted to 7.4 with Tris-base. The voltage dependent Ca^{2+} channels were blocked by using 0.2 mmol/L $CdCl_2$. Intracellular solution containing 140 mmol/L KCl, 10 mmol/L

EGTA, 3 mmol/L MgATP, 10 mmol/L HEPES, and 10 mmol/L glucose. The pH was adjusted to 7.2 with Tris base. The internal recording solution was filtered through a Millipore 0.2 μm syringe filter before use and frozen till the experiment. Both external and internal solutions were of analytical grade. DTT, TTX, EGTA, MgATP, Na₂GTP, HEPES, CsCl, TeCl, collagenase and bovine serum albumin were obtained from Sigma.

Preparation of cells

Animals were provided by the Experimental Animal Center of Medical College of Xi'an Jiaotong University. All procedures were carried out in accordance with the United States Public Health Services Guide for Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of Xi'an Jiaotong University. The smooth muscle cells were freshly dispersed as described previously^[7]. Briefly, male Sprague-Dawley adult rats weighing 120-150 g were anesthetized with ether. The mesenteric arterial branches were dissected. The connective tissue and the endothelium were carefully removed under microscope (XTZ-E, Shanghai Shangguang Microscope Co., Shanghai, China) and the smooth muscle was cut into 1-2 mmol/L sections. The tissues were preincubated with oxygenated Ca²⁺-free PSS for 40 min at 18-22°C, then incubated in oxygenated Ca²⁺-free PSS containing 0.3% collagenase I, 0.3% bovine serum albumin, and 6 mg DTT for 25-30 min at 32°C. The tissues were washed three times with oxygenated Ca²⁺-free PSS, and then gently agitated with a blunt-tipped glass pipette until the solution became cloudy. The debris was then removed with a fine 200 μm nylon mesh. The cell suspension was stored at 4°C and used within 5 h after cell harvesting.

Electrophysiological recordings

The smooth muscle cells were transferred to a recording chamber mounted on an inverted microscope (DMIRB, Leica, Germany) for 15 min. Only spindle-shaped cells were selected for experimentation. Electrophysiological recordings were performed with standard whole cell voltage-clamp technique^[8]. Recording pipettes were made from borosilicate glass capillaries using a puller (model p-97, Sutter Instrument, Novato, CA) and heat-polished to allow gigaohm seal formation. The resistance of the recording pipettes filled with internal recording solution was 3-6 M Ω in bath solution. The junction potential between the patch pipettes and bath solution was nullified immediately before G Ω seal formation. Cell capacitances were read from the potentiometer to set transient capacitance to zero. This value was used to calculate the cell size. After pipette and cell transient capacitance were compensated with the appropriate controls on the amplifier, the membrane was ruptured with gentle suction to obtain the whole cell voltage-clamp configuration. Series resistance was routinely compensated by 60%-70% and monitored periodically. Voltage-gated Ca²⁺ currents and delayed rectifier potassium currents (I_{KD}) were recorded as previously described^[9]. Electrophysiological recordings were performed at room temperature (22-25°C).

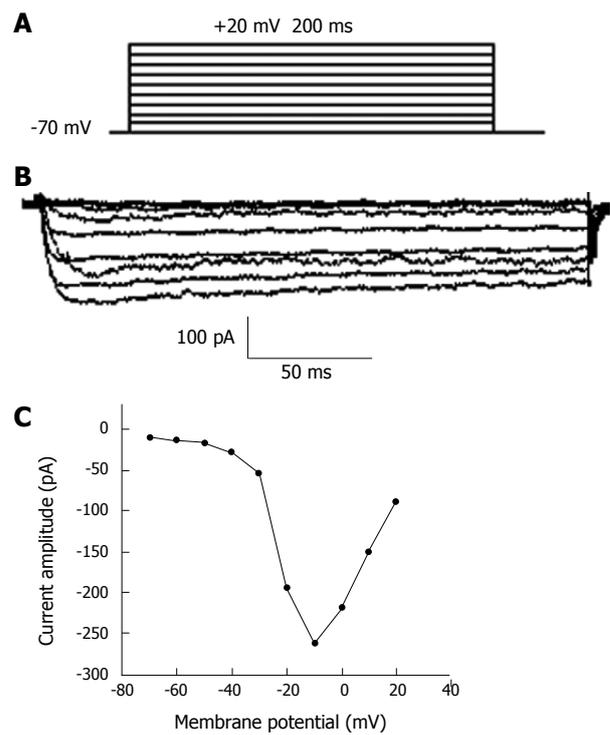


Figure 1 Voltage-gated calcium currents. **A:** Pulse protocols for recording voltage-gated Ca²⁺ current; **B:** Raw traces of calcium currents elicited in a freshly isolated smooth muscle cell; **C:** Current-voltage relation of calcium currents with isolated smooth muscle cells.

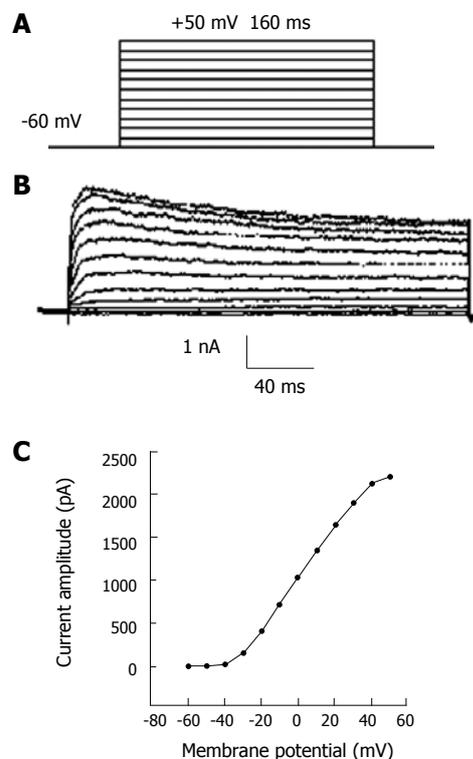


Figure 2 Delayed rectifier potassium currents. **A:** Pulse protocols for recording K⁺ current; **B:** Raw traces of K⁺ currents elicited in a freshly isolated smooth muscle cell; **C:** Current-voltage relation (I-V curve) of K⁺ currents with isolated smooth muscle cells.

Statistical analysis

All data were analyzed by pCLAMP CLAMPFIT proce-

dures (Axon Instrument, USA). Current-voltage relations of voltage-gated channels were plotted by Origin 6.0 software (Microcal Software, USA).

RESULTS

Voltage-gated calcium currents

Voltage-gated Ca^{2+} currents were elicited by 200 ms depolarizing potential from -70 mV to +20 mV with an increment of 10 mV. At 5 s intervals, a holding potential was -70 mV (Figure 1A). The example of raw Ca^{2+} current traces for isolated smooth muscle cell is shown in Figure 1B. No significant inward current was observed until the depolarizing step reached -40 mV. Currents reached maximum amplitude at approximately -10 mV in each case, and became small with stronger depolarization. The current amplitude in dependence of the potential could be shown as an I-V curve (Figure 1C). The maximal current peak amplitude of the curve was -228.64 pA.

Delayed rectifier potassium current

I_{Kd} was elicited by 160 ms depolarizing pulses (-60 mV to +50 mV) at 2 s intervals, from a holding potential of -60 mV (Figure 2A). Figure 2B shows a series of raw current traces evoked. The outward currents showed a slow activation and inactivation during depolarizing pulses, and increased with increasing test potentials. The currents were sensitive to TeaCl , a potassium current blocker. The currents recorded under the condition were outward delayed rectifier potassium currents. Current-voltage (I-V) curve of I_{Kd} was obtained by plotting the currents evoked against test pulse (Figure 2C). The maximal current peak amplitude of the curve was 2216.75 pA.

DISCUSSION

Theoretically, isolation with enzyme irreversibly digests the protein-including ion channels in cell membrane, which could not be excluded with our method. Compared with methods reported previously^[5,6], the present methods have the following advantages. The isolating process is simple, fast and easy to manipulate using a single enzyme instead of several enzymes. The yield of the freshly isolated smooth muscle cells with high quality is increased markedly, leading to a $\text{G}\Omega$ seal. The activity and dose of enzyme could influence the cells' quality and quantity. Excessive enzyme destroys the normal structure of cells and causes swelling of cells. The membranes of the remanent

living neurons are easy to break before $\text{G}\Omega$ seal easily, which is the key to successful patch clamp experiments. On the other hand, the enzyme is not enough to disrupt the connection between cells. When pipetted, the sections are difficult to disperse. Incubation of sections is beneficial to their recovery. Careful and slow trituration of tissue sections is necessary and air bubble should be avoided to prevent cell damage.

In conclusion, Ca^{2+} and K^{+} channel currents can be successfully recorded in acutely isolated smooth muscle cells. Our method can dissociate smooth muscle cells from rat mesenteric arterial branches, and is a powerful tool for functional analysis at the level of individual smooth muscle cells.

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Acute liver failure due to natural killer-like T-cell leukemia/lymphoma: A case report and review of the Literature

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Abstract

Acute liver failure (ALF) is a medical emergency requiring immediate evaluation for liver transplantation. We describe an unusual case of a patient who presented with ascites, jaundice, and encephalopathy and was found to have ALF due to natural killer (NK)-like T cell leukemia/lymphoma. The key immunophenotype was CD2+, CD3+, CD7+, CD56+. This diagnosis, which was based on findings in the peripheral blood and ascitic fluid, was confirmed with liver biopsy, and was a contraindication to liver transplantation. A review of the literature shows that hematologic malignancies are an uncommon cause of fulminant hepatic failure, and that NK-like T-cell leukemia/lymphoma is a relatively recently recognized entity which is characteristically CD3+ and CD56+. This case demonstrates that liver biopsy is essential in diagnosing unusual causes of acute liver failure, and that infiltration of the liver with NK-like T-cell lymphoma/leukemia can cause acute liver failure.

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Key words: Liver failure; Liver transplant; Natural killer cell

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INTRODUCTION

Acute liver failure (ALF) is a medical emergency,

and while liver transplantation can be life saving, it is imperative to perform a thorough evaluation to screen for potential contraindications^[1]. We describe an unusual case of a patient who developed ALF due to natural killer (NK)-like T-cell leukemia/lymphoma, a diagnosis with important implications, as it is a contraindication to liver transplantation. While a review of the literature shows that previously published case series have found this rare subtype of T-cell malignancy to involve the liver, we believe that this is the first report of it presenting as ALF.

CASE REPORT

A previously healthy 63 year-old man was admitted for three mo of fatigue, one mo of increasing abdominal girth and peripheral edema, two weeks of jaundice, and one week of confusion. He noted recent fevers, anorexia, weight loss, and day-night reversal. He had never received a blood transfusion or used intravenous or intranasal drugs. He had no tattoos or recent travel. He did not take prescription or over-the-counter medications, or nutritional or herbal supplements. He admitted using alcohol heavily in the past, but had been sober for more than 10 years. His family corroborated this information. There was no family history of liver disease.

On physical examination he was jaundiced and had findings consistent with hepatic encephalopathy including slurred speech and asterixis. He had moderate ascites, peripheral edema, and scattered spider angiomas. The liver was normal in size, but splenomegaly was detected.

Routine laboratory tests revealed hyponatremia, hypoalbuminemia, hyperbilirubinemia, thrombocytopenia, and prolonged prothrombin time not due to vitamin K deficiency (Table 1). The white blood cell count was $5.1 \times 10^9/L$ with atypical lymphocytes comprising 53% of the differential (Figure 1). Other diagnostic testing found a negative toxicology screen, 90% iron saturation, negative serologies for hepatitis A virus, hepatitis B virus, hepatitis C virus, human immunodeficiency virus, and human T-cell lymphotropic virus (HTLV) types I and II. Serologies were also negative for anti-nuclear antibodies, anti-smooth muscle antibodies, and anti-mitochondrial antibodies. Serum protein electrophoresis, alpha-1 anti-trypsin levels, ceruloplasmin levels, and alpha-fetoprotein were normal. Serum ammonia level was not measured. Abdominal ultrasound showed a normal liver size with heterogeneous echotexture, splenomegaly, and patent vasculature. Head CT scan excluded mass lesions and infiltrating disease.

Table 1 Laboratory results

Test	Result (normal range)
Sodium	128 mmol/L (135-145)
Creatinine	1.1 mg/dL (0.8-1.4)
AST (SGOT)	85 U/L (19-55)
ALT (SGPT)	40 U/L (19-72)
Total bilirubin	8.4 mg/dL (0.0-1.2)
Direct bilirubin	7.1 mg/dL (0.0-0.4)
Alkaline phosphatase	138 U/L (38-126)
GGT	80 U/L (13-68)
Serum albumin	1.7 g/dL (3.5-5.0)
Ascitic fluid albumin	0.5 g/dL (unspec)
White blood cell count	5.1 × 10 ⁹ /L (4.5-11)
neutrophils (%)	47
monocytes (%)	0
lymphocytes (%)	53 with atypical forms present
eosinophils (%)	0
Hematocrit (%)	33.3 (41-53)
Platelet count	31 × 10 ⁹ /L (150-440)
Prothrombin time	20 s (11-14)
International normalized ratio (INR)	1.7

Diagnostic paracentesis at a site in the left-lower-quadrant demonstrated a serum albumin-ascites gradient of 12 g/L, 12 475 red blood cells (RBCs)/mm³ and 1875 white blood cells (WBCs)/mm³ with a differential of 1% neutrophils, 4% monocytes and 95% lymphocytes. Because the lymphocytes were described as atypical with mitotic figures, a repeat paracentesis was performed at a right-lower-quadrant site. This revealed 1550 RBCs/mm³ and 250 WBCs/mm³ with the same differential and atypical cells. A sample of the ascitic fluid was sent for cytology (Figure 2). The peripheral blood flow cytometric immunophenotypes were as follows: CD2+, CD3+, CD7+, CD56+, CD4-, CD5-, CD8-, CD57-, and CD16-. A bone marrow biopsy revealed the same findings. Cytogenetic analysis of the bone marrow aspirate revealed the following karyotypes: 43, X, -Y, add (4) (q35), -5, dic (6;19) (q22; q13.4), -10, -11, -13, -14, -16, -18, add(22)(p11), +6 mars.

Since the patient's coagulopathy prohibited percutaneous liver biopsy, transjugular liver biopsy was performed for definitive diagnosis. There was no evidence of cirrhosis, but there was diffuse hepatic infiltration by a malignant lymphoid population (Figures 3A and 3B) which was immunohistochemically stained as follows: CD3+, CD20-, Epstein-Barr virus (EBV)-, granzyme B+, T1A-1+, and TdT-. The paraffin block of the liver biopsy was analyzed for a T-cell receptor (TCR) gamma gene rearrangement by polymerase chain reaction, and there was no evidence of a clonal TCR gamma gene rearrangement.

The patient was diagnosed with NK-like T-cell lymphoma/leukemia as the cause of liver failure on hospital d 4. This was deemed a contraindication to transplantation. His course was complicated on d 3 by bleeding duodenal ulcers amenable to standard endoscopic treatment. On d 4-6, high-dose methylprednisolone failed to induce remission, and his hepatic synthetic function and mental status worsened. On d 10 and 11, a salvage regimen

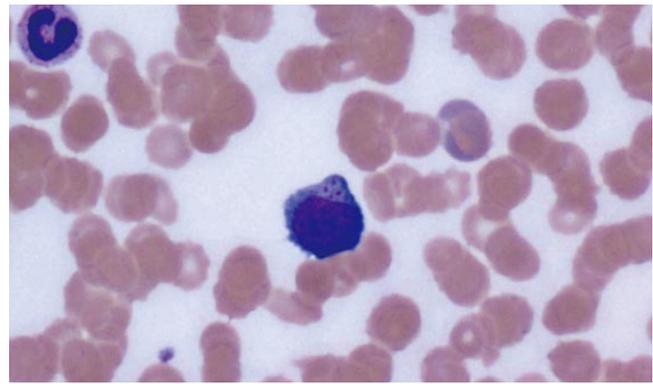


Figure 1 Peripheral blood smear-high power (1000 ×) view of atypically large lymphoid cells with blastic chromatin and abundant cytoplasm containing fine azurophilic granules.

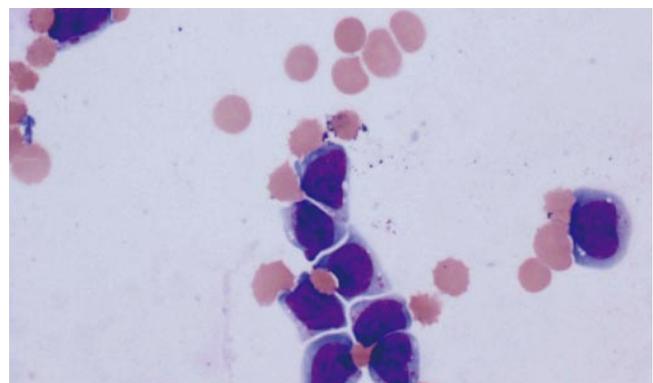


Figure 2 Ascites cell-block- high power (600 ×) view of large lymphocytes with molding, convoluted nuclear membranes, dense chromatin, and abundant cytoplasm. Features are supportive of possible T-cell morphology.

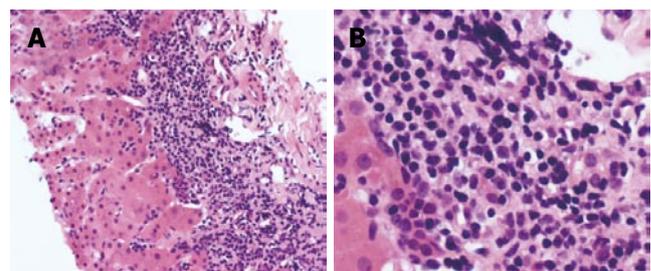


Figure 3 Liver biopsy-low power (200 ×) view of the liver parenchyma infiltrated by a malignant lymphoid population composed of intermediate-sized to large atypical lymphocytes (A) and liver biopsy-high power (1000 ×) view of the liver parenchyma showing intermediate-sized to large atypical lymphocytes with very irregular nuclear contours and dense nuclear chromatin (B).

of gemcitabine was unsuccessful in inducing remission. On d 12, neutropenia, fever, and hypotension developed, and the patient's jaundice and encephalopathy progressed. The patient expired on hospital d 12.

DISCUSSION

The diagnosis of acute liver failure is a medical emergency because mortality is high without liver transplantation^[1]. However, thorough evaluation is mandatory to exclude contraindications to liver transplantation such as

underlying malignancy^[1,2].

Hematologic malignancies often incidentally involve the liver, but they uncommonly present as acute liver failure^[3,4]. When they do so, however, they carry a poor prognosis^[5]. Acute leukemias, Hodgkin's disease, non-Hodgkin's lymphoma, and transformation of chronic leukemias have all been noted in case reports or case series to present as hepatic failure^[5-20]. Infiltration of the liver and subsequent hepatic dysfunction specifically by T-cell leukemias and lymphomas are more unusual, though it has been reported in both the pediatric^[21,22] and adult patients^[23-29].

Natural killer cells, typically identified morphologically as large granular lymphocytes, are recognized for their role in cell-mediated immunity^[30]. They have characteristic immunophenotypes (CD3-, CD56+) which distinguish them from T cells (CD3+, CD56-). Other markers such as CD8 and the additional so-called NK-cell antigens (CD16 and CD57) are variably present^[31-32]. Over the past two decades, it has been noted that NK cells undergo clonal expansion and malignant transformation^[33]. This is believed to be a rare event, accounting for only a small fraction of all T-cell malignancies, which themselves are thought to comprise approximately just 15% of all non-Hodgkin's lymphoma^[31,34]. NK-cell leukemia/lymphomas are categorized as either immature (and more aggressive) or mature (with an indolent course), and more commonly affect the nasopharynx and sinuses though they have also been reported to involve the skin, mucosa of the gastrointestinal (GI) tract, testes, kidneys, and orbit^[31,35]. They are seen more frequently in Asia, Mexico, and South America, ostensibly due to associations with EBV and HTLV^[36-38]. Of note, while they are commonly associated with hepatosplenomegaly they have not been reported to present with acute liver failure^[31,39-41].

In contrast to NK cells, NK-like T-cells expressing features of both T cells and NK cells, are defined by a CD3+, CD56+ immunophenotypes, and tend not to be associated with EBV^[31]. The first reports of NK-like T-cell leukemias and lymphomas^[42,43] have described presentations including typical "B" symptoms such as fever, chills, night sweats and weight loss, as well as lymphadenopathy and splenomegaly. Disease involves the spleen, blood, marrow, GI tract, lung, kidneys, and liver, but none of the patients presented with frank liver dysfunction. In most instances, the disease is aggressive and rapidly fatal, regardless of treatment regimen. A large series identified 49 Chinese patients with extra-nasal CD56+ disease, 34 of whom are also CD3+^[38]. Of the 5 patients with liver involvement, all had reactive hemophagocytic syndrome but none presented with hepatic failure. Of the 29 patients with follow-up information available, 24 died in a median time of 3.5 mo. A recent phenotypic analysis of 408 Japanese cases of peripheral T/NK cell lymphoma had not described any with liver failure^[44].

In this paper we present the clinical course, immunophenotype, and cytogenetics of what we believe to be the first reported case of a patient with acute liver failure due to NK-like T-cell leukemia/lymphoma. This has implications beyond the rarity of this particular patient's condition. Because this patient had a history of alcohol consumption, his liver disease might have been

ascribed to alcoholic hepatitis from a relapse of alcohol use. However this case illustrates the importance of fully analyzing unexpected findings. The atypical lymphocytes seen on the peripheral smear and in the ascitic fluid cell count differential were not attributed to a reactive process. Rather, they led to the transjugular liver biopsy which is essential in making the diagnosis, and underscores the role of and importance of liver biopsy when the etiology of liver failure is unknown.

In conclusion, a hematologic malignancy infiltrating the liver, although rare, is a contraindication to liver transplantation. It is mandatory to exclude such processes in the correct clinical context. In our case, liver biopsy allowed prompt diagnosis and recognition of NK-like T-cell leukemia/lymphoma as the cause of acute liver failure.

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Situs inversus abdominus and malrotation in an adult with Ladd's band formation leading to intestinal ischaemia

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Abstract

Situs inversus abdominus with rotational anomaly of the intestines is an extremely rare condition. Although intestinal malrotation has been recognized as a cause of obstruction in infants and children and may be complicated by intestinal ischaemia, it is very rare in adults. When it occurs in the adult patient, it may present acutely as bowel obstruction or intestinal ischaemia or chronically as vague intermittent abdominal pain. Herein, we present an acute presentation of a case of situs inversus abdominus and intestinal malrotation with Ladd's band leading to infarction of the intestine in a 32 year old woman.

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Key words: Situs inversus; Malrotation; Ischaemia; Intestine

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INTRODUCTION

Situs inversus abdominus is an extremely rare condition when it occurs in adults. It may present with vague abdominal pain or with intestinal obstruction or with intestinal ischaemia. We describe a patient with situs inversus abdominus who presented to us acutely with intestinal ischaemia.

CASE REPORT

A 32-year-old woman presented to our emergency

department with severe cramping and generalized abdominal pain of 2 d duration. This cramping pain occurred every 2 to 3 h and was 30 to 40 min in duration. She vomited several times which was bilious in nature. Her last bowel movement was 2 d prior, and it was described as normal. Her past medical history included mitral valvuloplasty for rheumatic heart disease. She had also developed cardiac cirrhosis secondary to rheumatic heart disease. There was no history of previous abdominal surgery and denied alcohol or tobacco use.

On physical examination, the patient's vital signs were pulse 90, blood pressure 126/67, temperature 37°C, and respiratory rate was 16. The abdomen was not distended. She exhibited no peritoneal signs; however, mild diffuse tenderness to deep palpation was appreciated. Normal bowel sounds were present on auscultation. Her rectal examination was normal. Haemoglobin, white blood cell count, basic biochemistry panel and arterial blood gases were all within normal values. Abdominal X-rays did not reveal any features of obstruction. Chest radiography did not reveal any signs of perforation of a hollow viscus. A prompt computed tomography (CT) scan of the abdomen was performed which demonstrated situs inversus abdominus and free air intraperitoneally suggesting perforation of a hollow viscus (Figure 1). She was taken to theatre urgently for a laparotomy, which revealed malrotation of the intestines with complete situs inversus. The entire small bowel was found to be of doubtful viability and there was a presence of Ladd's band running from the ileocaecal valve to the duodenojejunal flexure around which the small bowel had rotated (Figure 2). The Ladd's band was divided which improved the perfusion of the bowel to a reasonable extent and a decision was made to pack the wound and have a second look laparotomy in the next 12 h. Postoperatively she was nursed in the intensive care unit where she developed endotoxic shock and multiple organ failure. At the second laparotomy, the entire small and the large bowel were infarcted which was deemed incompatible with life. A decision was made to close the abdomen and the patient died in the next few hours in the intensive care unit.

DISCUSSION

Situs inversus abdominus (SIA) is an uncommon anomaly with an incidence varying from 1 in 4000 to 1 in 20000 live births^[1]. Acute surgical emergencies complicating SIA are extremely rare in the adult and very few cases are reported in the English literature^[2,3]. Situs inversus was

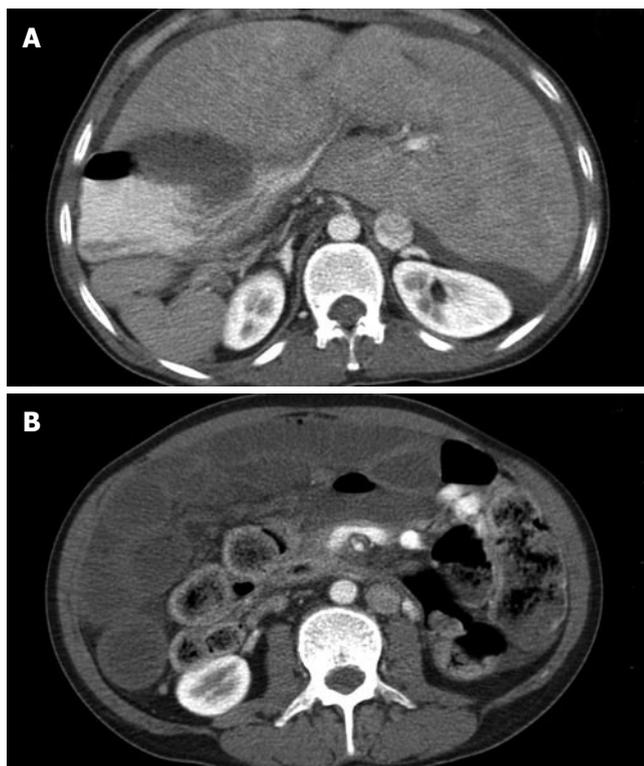


Figure 1 CT Scan imaging showing (A) situs inversus (note liver on the left and stomach on the right side) and (B) free intraperitoneal air.

first described by Aristotle in animals and by Fabricius in humans^[4]. Congenital anomalies of intestinal rotation are often seen in infants and children; however, they are uncommon in adults. Approximately 85% of malrotation cases present in the first 2 wk of life^[5].

A brief knowledge of intestinal embryology is necessary in order to understand the rotation of the intestine and its anomalies. The embryo's gut is in the form of a straight tube at the fourth week of intra-uterine life. Approximately during the fifth week, a vascular pedicle develops and the gut can be divided into foregut, midgut, and hindgut. The superior mesenteric artery supplies blood to the midgut. The midgut is defined by a rapidly enlarging loop with the superior mesenteric artery running out to its apex. The cephalad portion of the loop gives rise to the first six metres of small intestine, and the remainder of the loop forms the distal small bowel and colon up to the splenic flexure. Intestinal rotation primarily involves the midgut. The rotation of intestinal development has been divided into 3 stages. Stage I occurs in wk 5 to 10. It includes extrusion of the midgut into the extra-embryonic cavity, a 90° anti-clockwise rotation, and return of the midgut into the fetal abdomen. Stage II occurs in week 11 and involves further anti-clockwise rotation within the abdominal cavity completing a 270° rotation. This rotation brings the duodenal "C" loop behind the superior mesenteric artery with the ascending colon to the right, the transverse colon above, and descending colon to the left. Stage III involves fusion and anchoring of the mesentery. The caecum descends, and the ascending and descending colon attach to the posterior abdominal wall.

Intestinal anomalies can be classified by the stage of

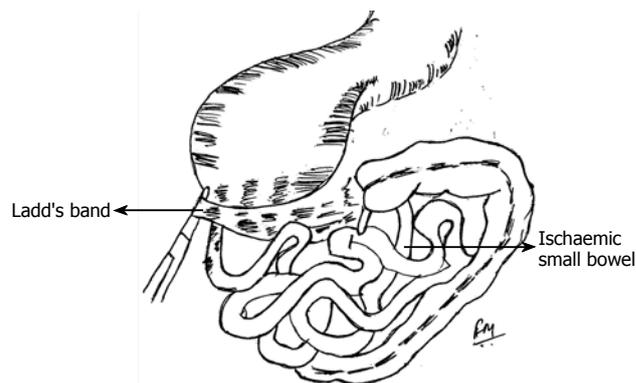


Figure 2 Schematic intraoperative findings show the presence of Ladd's band with small bowel ischaemia.

their occurrence^[6]. Stage I anomalies include omphaloceles caused by failure of the gut to return to the abdomen. Stage II anomalies include nonrotation, malrotation, and reversed rotation. Stage III anomalies include an unattached duodenum, mobile caecum, and an unattached small bowel mesentery.

Many remain asymptomatic through life and the anomaly is discovered only at autopsy. Some may present with chronic and unexplained abdominal discomfort, and even fewer may report acute episodes of agonizing abdominal pain. Symptoms can arise from acute or chronic intestinal obstruction that may be caused by the presence of abnormal peritoneal bands (Ladd's bands) or a volvulus.

Malrotation of the colon is estimated to occur in 0.2% to 0.5% of the adults and is significant clinically in only a small portion of them. There are no typical set of symptoms that are diagnostic of this clinical problem. The location of the pain may vary from epigastric to left upper quadrant. Often occurs postprandially and may last several minutes up to 1 h. Patients are frequently followed for a long period before undergoing surgery. The difficulty of diagnosis lies in both the absence of specific physical findings and the low adult frequency. Symptoms in the adult patient are often mistaken for irritable bowel syndrome, peptic ulcer disease, biliary and pancreatic disease, and psychiatric disorders^[7].

The diagnosis of rotational anomaly can be made by radiographic studies^[8]. In the absence of volvulus, a plain x-ray of the abdomen is of little diagnostic value. The absence of caecal gas shadow or the localization of small intestinal loops predominately in the right side should arouse the suspicion of malrotation^[9]. Malrotation can be diagnosed on CT by the anatomic location of a right-sided small bowel, a left-sided colon and an abnormal relationship of the superior mesenteric vessels. Nichols and Li described the abnormal position of the superior mesenteric vein (SMV) wherein the SMV was situated to the left of the superior mesenteric artery (SMA) instead of to the right on CT scan in patients with malrotation^[10].

The classic treatment for incomplete intestinal rotation is the Ladd procedure, which entails mobilization of the right colon, division of Ladd's bands and mobilization of the duodenum, division of adhesions around the SMA to broaden the mesenteric base, and an appendectomy. This

procedure was originally described by Ladd^[11].

In conclusion, adult presentation of situs inversus abdominus with intestinal malrotation is a difficult diagnosis because of the rare incidence of the disorder. These patients often present with chronic abdominal pain or with ischaemia as exemplified by our case. Diagnosis requires a high index of suspicion.

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CASE REPORT

Gastric metastasis by lung small cell carcinoma

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Abstract

Metastatic tumors of the gastrointestinal tract are rare. We describe a case of gastric metastasis due to primary lung cancer, revealed by an upper gastrointestinal endoscopy (UGIE). Haematogenous metastases to the stomach are a rare event. To our knowledge, only 55 cases have been described in the international literature. In these patients, the prognosis is very poor. We report herein a case of gastric metastasis by lung small cell carcinoma, with a review of the literature about this rare entity.

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Key words: Gastric metastasis; Lung cancer

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<http://www.wjgnet.com/1007-9327/12/4096.asp>

INTRODUCTION

Metastatic tumors of the gastrointestinal tract are rare^[1]. In most cases, the diagnosis is possible only at autopsy. To our best of knowledge, only 55 cases have been described in international literature so far^[2].

CASE REPORT

We describe a case of gastric metastasis due to primary lung cancer revealed by an upper gastrointestinal endoscopy (UGIE). In March 2005, a 63-year-old

Caucasian male was referred to our department because of fever (38°C), weight loss (5 kg in few months), epigastric pain, and constipation. He had an accidental chest trauma associated to right rib fractures 3 years back. He was a smoker of 20 cigarettes per day. At admission to our department, his general conditions were poor. Lymphadenopathy (> 3 cm) in the left scalenic region, and liver enlargement were evident. A mild anaemia (Hb 110 g/L), low platelet count ($40 \times 10^9/L$), and a marked increase of lactic dehydrogenase (LDH > 1000 U/L) were the most important blood biochemical alterations. A chest x-ray revealed a lung mass (> 5 cm in diameter) in the upper left hilar region. Multiple 1-3 cm secondary liver lesions scattered in both lobes, and with a typical "bull's eye" appearance were revealed by ultrasonography. An UGIE showed a 1.5-cm raised area, depressed on the tip with recent signs of haemorrhage in the gastric corpus, which was histologically identified as the metastasis of an undifferentiated small cell carcinoma (Figure 1). A total body computed tomography (CT) confirmed a left lung neoplasm associated to an infiltration of the left pulmonary artery, left upper bronchus stenosis, bulk mediastinal lymphadenopathies (> 3 cm), and bilateral pleural effusions. Liver secondaries were confirmed and multiple brain metastases were localised in the left parietal lobe and, bilaterally, in the occipital lobes. A total body bone scan showed diffuse bone involvement. A bronchoscopy and percutaneous biopsy of the left supraclavicular lymph node confirmed the diagnosis of diffuse poorly differentiated primary lung small cell cancer (Figure 2). The patient was provided only with a supportive care, considering his poor condition. He died one month later.

DISCUSSION

Haematogenous metastases to the stomach is a rare event^[3]. The most frequent tumors involved in secondary gastric sites are melanoma, breast, and lung cancer. Most patients with gastrointestinal metastases are asymptomatic^[1]. At autopsy, gastric metastases are present in 14% of all patients with lung cancer^[4]. Lung small cell carcinoma has a high incidence of vascular invasion^[3] and the gastric submucosal layer is always involved, followed by the mucosa, muscularis propria, and serosa^[5]. Complete inclusion of the metastasis within the gastric wall may be a sign of a primary tumor of another origin^[5].

Abdominal pain is the most frequent (80% of the cases) symptom in the symptomatic patient. Kadakia

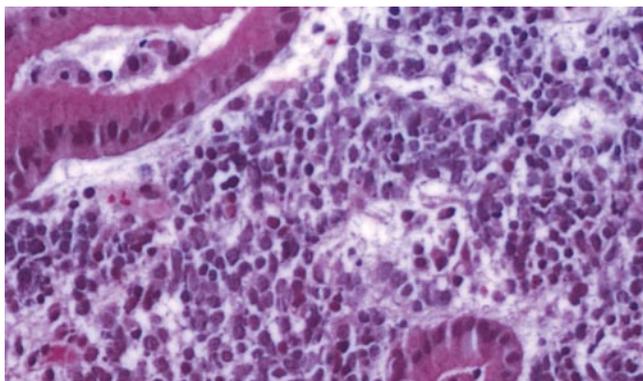


Figure 1 Middle view of small cell among gastric glands (x 20).

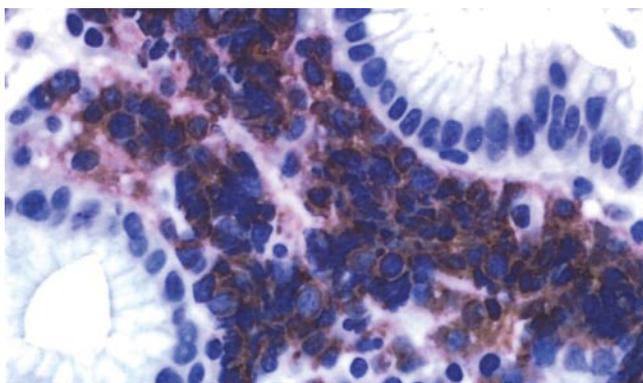


Figure 2 Diffuse positivity for synaptophysin (avidin-biotin-peroxidase complex method x 40).

et al^[1] also reported iron-deficiency anaemia and acute upper GI bleeding. Severe upper gastrointestinal bleeding, hypovolemic shock, and stomach perforation may be the cause of death of the patient. Differential diagnosis with lymphoma, ectopic pancreas, carcinoid tumor, eosinophilic granuloma, Kaposi's sarcoma and gastric ulcer is important^[3]. Gastric metastatic lesions have a particular endoscopic aspect, appearing as "volcano-like" or umbilicated on the tip^[3] (Figure 3). These lesions may present with three different appearances^[3]: (1) multiple nodules of variable size with a central ulcer; (2) submucosal, raised, and ulcerated at the tip and defined as "volcano-like"; and (3) raised areas without a central ulcer. In few cases, the lesions appear as polyps or raised plaques^[3]. It has been reported a high percentage of gastric metastases in the fundus and cardias^[4], but in our experience, the lesions are present in the gastric corpus region, which is in agreement with Kadakia *et al*^[1].

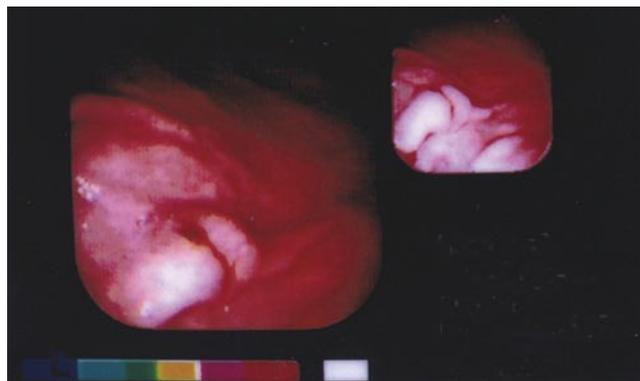


Figure 3 Typical endoscopic aspect of "volcano-like" lesion, suggestive of gastric metastatic lesion.

Hamatake *et al*^[6] described a rare case of solitary gastric metastasis from the lower left lung. This was a poorly differentiated adenocarcinoma diagnosed during UGIE for UGI bleeding, which resolved after gastrectomy and left lobectomy.

It is important to identify these lesions in order to prevent serious complications, such as gastrointestinal bleeding, viscus perforation, or gastric outlet obstruction^[4]. An upper gastrointestinal endoscopy should be recommended before starting chemotherapy^[5], as confirmed by Suzaki *et al*^[7] who described a case of gastric perforation during systemic chemotherapy for lung neoplasm due to metastasis^[7].

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CASE REPORT

Endoscopic management of intragastric penetrated adjustable gastric band for morbid obesity

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Abstract

This report describes a case of successful endoscopic management of intragastric penetrated adjustable gastric band in a patient with morbid obesity. The favorable course of the case described here demonstrates that adjustable gastric bands in the process of migration need not be removed surgically in patients who are asymptomatic.

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Key words: Morbid obesity; Adjustable gastric band; Complications; Endoscopy; Endoscopic therapy

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INTRODUCTION

Adjustable gastric banding is an established procedure for the treatment of obesity. Early complications associated with gastric banding included per operative bleeding, infection, and pneumonia. Late complications relate to the band or to the port system. The former include displacement, with resultant valve or pouch enlargement; gastric perforation; and band penetration. Port-associated complications include infection, disconnection, or

migration. These late complications usually are corrected by surgery^[1-9]. Penetration of a gastric band into the stomach lumen is a rare late complication. In almost all cases, a penetrated band must be removed surgically but, under certain circumstances, endoscopic intervention can be attempted. We here report a case of endoscopic management of intragastric penetrated adjustable gastric band.

CASE REPORT

A 47-year-old man with morbid obesity (weight: 113 kg, body mass index 43.1 kg/m²) underwent open implanted adjustable gastric band (November 1997). The post-interventional follow up showed a good weight loss (weight 96.6 kg). Ten months later (September 25, 1998) because of the patient was found to have unexplained weight gains (weight 108 kg), filling volume in the band was increased to 2.5 mL. This was, however, ineffective (weight 110 kg), and because of the patient reported the possibility to have complete and plentiful meals a port disconnection was suspected. The port was replaced under local anesthesia. On November 2001, the patient was found to have once again weight gains (weight 117 kg) because of a recurrent port disconnection. A new port was implanted under local anesthesia and a filling volume in the band was progressively increased to 3 mL. On August 2002 (weight 93.5 kg), the patient experienced a sub-cutaneous abscess at site of port implantation, due to port-associated infection. An upper GI endoscopy was unremarkable and because of the infection was unresponsive to antibiotics, the port was removed and re-implanted. However, a recurrent port-associated infection was diagnosed 1 mo later and the patient was scheduled to receive a monthly upper endoscopy because of the risk of penetration of the gastric band into the gastric lumen. Six months later (February 2003; 62 mo after the banding procedure) endoscopy demonstrated a partial penetration of the gastric band into the gastric lumen (Figure 1). Because of the patient was asymptomatic at that time, it was decided to await the complete intragastric band migration for an endoscopic attempt to remove the gastric band. Gastroscopy 13 mo later (March 2004), revealed almost total penetration of the band; only a small tissue bridge held the device to the gastric wall. With almost complete penetration of the band, an endoscopic approach was

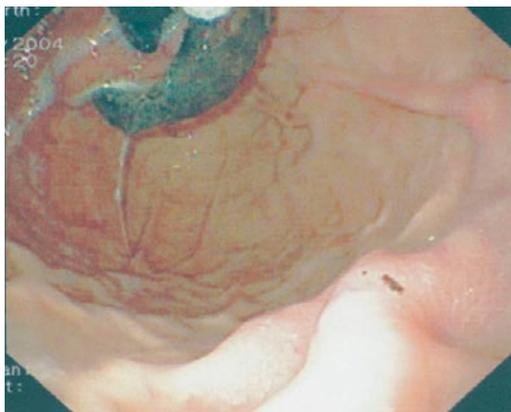


Figure 1 Endoscopic appearance of intragastric migration of band.



Figure 2 Endoscopic retrieval of the gastric band.

felt to be safe and feasible. An endoscopic attempt to remove the gastric band was therefore performed with surgical standby. The tissue bridge was injected with a dilute solution of epinephrine (1:10 000) and the tissue bridge then was resected step by step by using a needle-knife papillotome. After severing the tissue bridge, the band remained attached to the port system. Most of the silicone tube connecting the injection port with the gastric band was resected and the attached port was removed. The rest of the tube slipped into the stomach, the band lying freely within the gastric lumen. The band was cut by the means of the CBC360 AMI gastric banding cutter (C.J.Medical. Haddenham, Buckinghamshire, England) and finally retrieved endoscopically by using a polypectomy snare (Figure 2). The connecting tube was removed through little cutaneous incision. The post-interventional course was uneventful. A gastrografin swallow done postoperatively ruled out a leak, and patient was discharged 3 d later.

DISCUSSION

Penetration of a gastric band into the stomach lumen is a rare late complication^[2-9]. The suggested primary etiologic factor for the band penetration is pressure applied to the gastric wall. External pressure is applied either through chronic overfilling of the band or the inclusion of too much gastric wall at operation) Internal pressure is applied as a result of ingestion of excessively large food boluses early after operation. Another suggested factor is a rejection reaction against the silicon gastric band with subsequent circumferential fibrous contraction^[3,9]. The main symptoms that suggest dislocation or penetration of a gastric band are weight gain and epigastric pain or port-associated infection. The diagnosis is established by endoscopy or x-ray study. Because barium contrast radiography provides additional information on other complications of gastric banding, such as band slippage or pouch dilatation, this imaging study should be performed initially. Nonetheless, the final diagnosis of band migration is established by endoscopy^[4,5]. In almost all cases, a penetrated band must be removed surgically, and to our knowledge,

there are only two published case reports of endoscopic management of a penetrated gastric band^[10,11]. Whether a penetrated gastric band can be managed endoscopically depends on the degree of penetration. The risk of gastric perforation after endoscopic management is higher in patients with a small or a partial penetration of the band. Thus, the optimal candidate for endoscopic therapy should have an almost complete penetration of the band. In this group of patients, the risk of an iatrogenic perforation is relatively small. As demonstrated by the present case, endoscopic management of a penetrated gastric band is feasible under certain circumstances, specifically nearly complete penetration of the band into the stomach. The favorable course of the case described here demonstrates that adjustable gastric bands in the process of migration need not be removed surgically in patients who are asymptomatic. Complete migration can be awaited and the gastric band retrieved less invasively. Compared with operative removal, the procedure described above is expected to have fewer complications.

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Successful treatment for esophageal carcinoma with lung metastasis by induction chemotherapy followed by salvage esophagectomy: Report of a case

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Abstract

We here report a case of a 51-year-old man with lung metastasis from esophageal carcinoma that was initially treated by combination chemotherapy consisting of fluorouracil and nedaplatin. Because metastatic disease disappeared, salvage esophagectomy was performed. Although chest wall recurrence developed at the thoracotomy wound, prolonged survival of 48 months was achieved by local tumor resection and additional chemotherapy. This combination chemotherapy is regarded as a promising and considerable treatment for metastatic esophageal carcinoma.

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Key words: Esophageal carcinoma; Lung metastasis; Induction chemotherapy; Fluorouracil; Nedaplatin; Salvage esophagectomy

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INTRODUCTION

It has been reported that 18% of patients with newly

diagnosed esophageal carcinoma have distant metastatic disease at presentation, in which the lung is the third most common site and accounts for 20% of metastasis cases^[1]. The prognosis of these patients is extremely poor and the urgent establishment of effective treatment is essential. We here describe a successful treatment for esophageal carcinoma with lung metastasis by induction chemotherapy followed by salvage esophagectomy.

CASE REPORT

A 51-year-old man complained of dysphagia for over two months. Endoscopy and a computed tomography (CT) scan of the chest performed in an affiliated hospital showed a circumferential tumor in the middle thoracic esophagus with bilateral multiple pulmonary nodules. He was referred to our hospital for further examination and treatment, and admitted on May 24, 2000. On physical examination, lymph nodes were palpable in the left supraclavicular fossa. Laboratory data including tumor markers were all within normal limits. A chest X-ray showed multiple small nodules in the bilateral lung. A barium meal study revealed an ulcerative tumor, 20 cm in length, mainly located in the middle and lower thoracic esophagus extending to the upper thoracic esophagus (Figure 1A). Endoscopy disclosed a protruding tumor 25 cm distal from the dental arch followed by a circumferential ulcerative tumor between 30 cm and 38 cm distal from the dental arch. A biopsy specimen histologically proved that it was a moderately differentiated squamous cell carcinoma. A CT scan of the neck through the abdomen demonstrated bilateral supraclavicular lymph node swelling and a total of 8 small nodules in the bilateral lung up to 1 cm in size with clear boundary (Figure 2A). Based on these findings, he was diagnosed with unresectable thoracic esophageal carcinoma with metastases to non-regional lymph nodes and lung, classified as stage IVB according to the TNM classification of the International Union Against Cancer (UICC)^[2]. With his written informed consent, he was registered with the Japan Clinical Oncology Group (JCOG) Trial (JCOG 9905), which was a phase II study of nedaplatin (NDP) and 5-fluorouracil (5-FU) in metastatic squamous cell carcinoma of the esophagus. Each course of chemotherapy consisted of 140 mg of NDP on d 1 and 1250 mg of 5-FU with continuous infusion on d 1-5.



Figure 1 A barium meal study before (A) and after (B) chemotherapy. More than 50 % decrease in total tumor size was demonstrated during chemotherapy, defined as partial response according to the guidelines of the Japanese Society for Esophageal Diseases^[3].

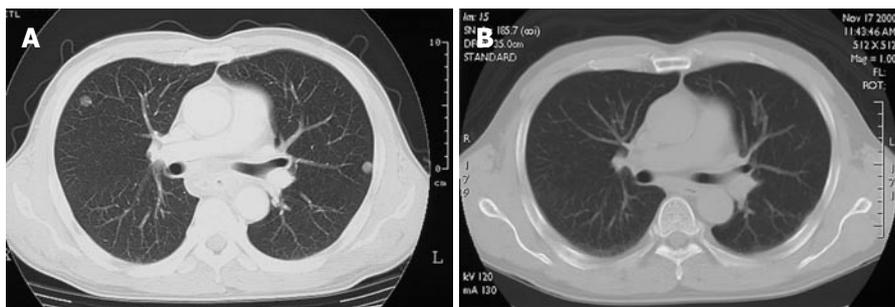


Figure 2 A chest computed tomography scan before (A) and after (B) chemotherapy. Complete disappearance of the multiple nodules in the bilateral lung was revealed during chemotherapy, defined as complete response^[3].

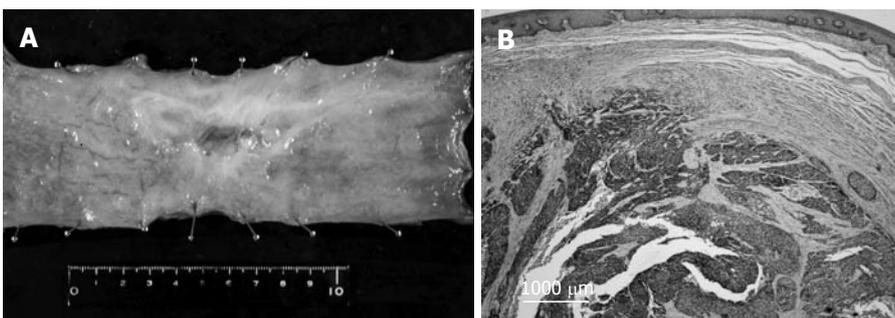


Figure 3 Resected specimen showing a 78 mm × 60 mm superficially invasive tumor (A) and histological examination showing foci of poorly-differentiated squamous cell carcinoma scattered in the submucosal layer (B) (HE, X 20).

Courses were repeated every 4 wk, and a total of 4 courses were performed. Hematological and non-hematological toxicities that developed during chemotherapy were grade 1 leukocytopenia and grade 3 anorexia, nausea, and stomatitis, respectively, as judged by the Common Toxicity Criteria of the National Cancer Institute (NCI-CTC) Version 2.0. After the completion of the 4th course of this combined chemotherapy, a CT scan revealed that multiple nodules in the bilateral lung disappeared (Figure 2B). No lymph node swelling was seen on the CT scan. On barium meal study, an irregular and slightly stenotic lesion, only 4 cm in length, was demonstrated in the middle thoracic esophagus (Figure 1B). A residual esophageal tumor was, however, endoscopically apparent as a slightly depressed lesion with a Lugol unstained area, which was histologically confirmed. Salvage esophagectomy was taken into consideration because the combined chemotherapy downstaged the patient's condition to that of a resectable disease.

On November 20, 2000, the patient underwent extended radical esophagectomy with a three-field lymphadenectomy, and reconstruction with a retrosternal gastric roll. No metastatic lesion was macroscopically found in the right lung during surgery. The resected specimen showed that the superficially invasive tumor was 78 mm × 60 mm in size (Figure 3A). Histological examination from the resected specimen showed foci of poorly-differentiated squamous cell carcinoma scattered in the submucosal layer (Figure 3B). No metastatic node was histologically identified. The histopathological response to chemotherapy was classified as grade 2 (moderately effective) according to the

guidelines of the Japanese Society for Esophageal Diseases^[3]. Consequently, R0 resection, according to the residual tumor classification of the UICC-TNM classification, was carried out^[2].

The patient had an uneventful postoperative course and was discharged 26 d after surgery. He was followed up at our outpatient department without any additional adjuvant therapy.

Chest wall recurrence probably due to tumor cell implantation developed at the thoracotomy wound nine months after esophagectomy. Because no other recurrence was detected, tumor resection was performed for local control. R1 resection was confirmed, as positive involvement of poorly-differentiated squamous cell carcinoma with the external surface of the resected specimen was histologically proved. Thereafter, the patient received two cycles of additional combined chemotherapy consisting of NDP and 5-FU, and was alive and well with no evidence of recurrence 48 mo after the second surgery.

DISCUSSION

The prognosis of patients with stage IVB esophageal carcinoma is extremely dismal. The 5-year survival rate of such patients after esophagectomy reported by the Japanese Society for Esophageal Diseases is 5.5%^[4]. The stage IVB disease comprises distinct two subgroups: one with distant organ metastasis and the other with non-regional nodal metastasis^[2]. Because esophagectomy has been mainly performed in the latter, overall survival of the disease is supposed to be even worse. Kato *et al*^[5] reported that the

3-year survival rate for esophageal cancer patients with distant organ metastasis is only 0.3%. Patients with distant organ metastasis are usually in poor condition at the time of presentation and receive only palliative treatment, radical treatment is selected only for patients in relatively good condition.

Surgery should be taken into consideration when complete resection of both primary and metastatic tumors is possible. In many cases, however, complete resection is impossible because multiple metastatic tumors are often detected in one or more organs. Although there are some reports on successful resection of esophageal carcinoma combined with liver metastasis^[6,7] or adrenal metastasis^[8], these cases seem to be exceptional.

Chemoradiotherapy is often performed in patients with non-regional nodal metastasis by extending the irradiation field^[9,10]. Nakada *et al.*^[11] reported that complete regression of esophageal cancer with concomitant liver metastasis and a long-term survival of 38 mo is achieved by concurrent chemoradiotherapy including metastatic lesions in the irradiation field. Except for these unusual cases, chemoradiotherapy is regarded as locoregional treatment in principle and should not be radical treatment for patients with distant organ metastasis.

Systemic chemotherapy is still widely accepted as a standard treatment for patients with distant organ metastasis. It is well known that squamous cell carcinoma of the esophagus is responsive to chemotherapy. Tumor reduction by at least 50% may occur in 35%–55% of patients with metastatic disease who receive cisplatin (CDDP)-based combination chemotherapy^[12]. Although chemotherapy can palliate symptoms in many patients, the response to chemotherapy typically lasts no longer than a few months and the median survival time of these patients is less than one year. To our knowledge, there is no other case in which an esophageal cancer patient with stage IV disease is down-staged by systemic chemotherapy, undergoes salvage esophagectomy for the residual esophageal tumor, and survives more than 4 years.

NDP is a second-generation platinum complex that has a characteristic property of being approximately 10 times as soluble in water as CDDP. Therefore, NDP is considered to have more pronounced activity against solid tumors and less nephrotoxicity and gastrointestinal toxicity than CDDP^[13]. A phase II study for metastatic squamous cell carcinoma of the esophagus using a combination of NDP and 5-FU has been conducted as a JCOG trial (JCOG 9905) in expectation of preferable anti-tumor effects and toxicities compared with that of CDDP and 5-FU^[14]. As a result, the overall response rate is 39.5% and 33.3% in

metastatic lung disease^[14]. Based on these results and the current case, combined chemotherapy consisting NDP and 5-FU is regarded as a promising and considerable treatment for metastatic squamous cell carcinoma of the esophagus.

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CASE REPORT

Sonographic findings of acute appendiceal diverticulitis

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Abstract

Preoperative images of acute appendiceal diverticulitis are rarely reported because of the difficulty of distinguishing appendiceal diverticulitis from other iliocecal diseases like acute appendicitis or cecal diverticulitis. We report a case of preoperatively diagnosed acute appendiceal diverticulitis. A 30-year-old female with a presumptive diagnosis of acute appendicitis from history and physical examination was admitted to our hospital. Ultrasound sonography showed inflamed appendiceal diverticula and inflammatory changes of the surrounding tissue. The swollen appendix was detected but its findings were slightly different from those of typical acute appendicitis in the following points. One difference was the thickened wall of the appendix, the other difference was the presence of air in the appendix. The patient underwent appendectomy and the pathological specimen revealed inflammatory changes of diverticula within the appendix.

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Key words: Appendiceal diverticulitis; Sonography; Preoperative diagnosis

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INTRODUCTION

Acute appendiceal diverticulitis is an uncommon clinical entity. Some patients treated for presumed acute appendicitis have acute vermiform appendiceal diverticulitis. Almost all acute appendiceal diverticulitis are found after appendectomy because history and physical findings of appendiceal diverticulitis closely resemble those of acute appendicitis. The preoperative images are not so effective

in detecting an appendiceal diverticulum. Here, we report a case of acute appendiceal diverticulitis diagnosed preoperatively by ultrasound sonography.

CASE REPORT

A 30 year-old female was admitted to our hospital for abdominal pain, slight fever and mild anorexia. The present episode of abdominal pain began four days before admission. At first, she felt epigastralgia and by the time she arrived at the hospital the pain shifted to the right lower abdomen. Physical examination showed a body temperature of 37.4°C and localized tenderness in the right lower quadrant of abdomen. No rebound tenderness was observed. Laboratory studies demonstrated a white blood cell count of $11.0 \times 10^9/L$. Abdominal roentgenogram showed no remarkable abnormality except for a small amount of intestinal gas. Abdominal ultrasound sonography, with a 7-MHz linear-array transducer (GE LOGIC 7), demonstrated an enlarged swollen appendix with a cross section diameter of 10 mm and multiple hypoechoic small lateral pouch-like projections (Figure 1). The surrounding was enclosed by pouch like projections in the hyperechoic lesion which was thought to be caused by inflammatory changes of the mesoappendix. Wall thickening of appendix was more prominent than that seen in typical appendicitis (Figure 2). We made a diagnosis of acute appendiceal diverticulitis. The patient underwent an emergent appendectomy. The resected appendix was 14 cm long and 1.0 cm in diameter with multiple diverticula. One of the diverticula was surrounded by inflammatory tissue (Figure 3). Microscopic study revealed a pseudodiverticulum with inflammation in its wall and mesoappendix. She had an uneventful recovery and was discharged two days after surgery.

DISCUSSION

Appendiceal diverticulum is an uncommon clinical entity, with an incidence of 0.2%-1.5% in surgical pathologic specimens^[1]. Its preoperative images have been rarely reported. Place *et al*^[2] have reported abdominal CT findings of appendiceal diverticulitis but they could not distinguish it from cecal diverticulitis preoperatively. Macheiner *et al*^[3] have also reported sonographic features of diverticulitis of the appendix vermiformis. According to their reports, a hypoechoic, inflamed diverticulum is surrounded by echogenic fatty tissue. This description is the same as our sonographic findings of acute appendiceal diverticulitis.

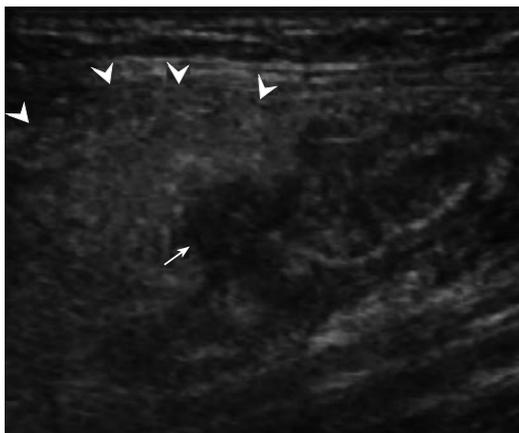


Figure 1 Longitudinal view of the appendix. A hyperechoic mass (arrowheads) including hypoechoic lateral pouch like lesion (arrow) is observed. Hypo lesion is appendiceal diverticula, and hyperechoic mass is inflamed adipose tissue (= mesoappendix).

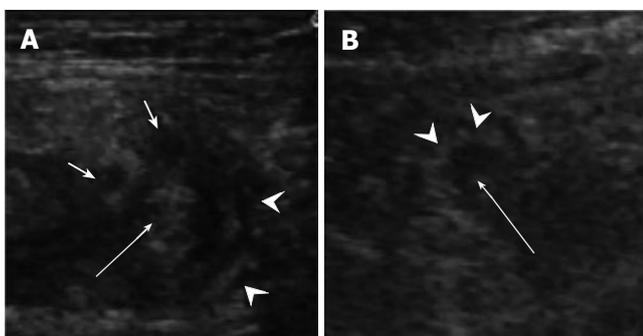


Figure 2 Cross section of acute appendiceal diverticulitis (A) in the present case and acute suppurative appendicitis (B) in another case. Layer structure (A: arrowheads) was found in appendiceal diverticulitis because of all inflamed layers, and inside echogenic (A: arrow) which means containing air. Multiple lateral hypoechoic projections (= diverticula) were observed. In comparison, an echogenic ring (B: arrowheads) was observed in acute appendicitis showing mucosal and submucosal inflammation, and an echo free space filled with inflammatory fluid inside it.

Other than these findings, we found different appendiceal features of acute appendiceal diverticulitis from those of

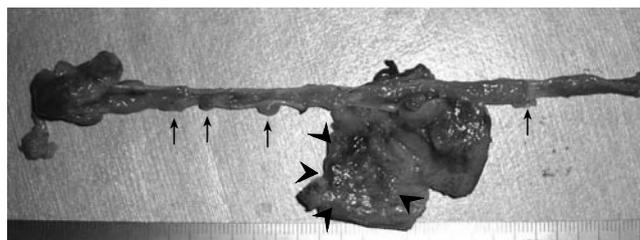


Figure 3 Multiple diverticuloses in the resected appendix (arrows) and peri-appendiceal inflammation (= diverticulitis) in 5 cm from the tip (arrowheads).

typical acute appendicitis (Figure 2). In typical suppurative acute appendicitis, edematous mucosa is observed as a cross-sectional ring of strong echo and inside it there is an echo free space filled with fluid. In comparison, the appendix wall layers are thickened and echogenic in appendiceal diverticulitis because of air in it. These findings strongly support that the main inflammation is in the appendiceal wall itself rather than in the actual appendix.

Diverticular disease of the appendix has four variations^[4], namely appendiceal diverticula without inflammation, acute appendicitis with diverticula, acute appendiceal diverticulitis with acute appendicitis, and acute diverticulitis. The fourth variation is true acute appendiceal diverticulitis as the case presented here. Its sonographic features resemble those of colonic diverticulitis rather than acute appendicitis except for the enlarged appendix. We suggest that sonographic study is useful for the early diagnosis of acute appendiceal diverticulitis.

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CASE REPORT

Small bowel anisakiosis: A report of two cases

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Abstract

Small bowel stenosis is a serious complication of intestinal anisakiosis. The aim of this report is to investigate whether severe stenosis of the small intestine can be conservatively managed. We treated two patients with severe stenosis of the small intestine caused by anisakiosis. Surgical intervention was eventually performed on the 23rd and 35th in the hospital, respectively. Histopathological examination of the resected specimens revealed that the intestinal wall had been completely damaged by the inflammatory reaction of anisakiosis, and that the damage was irreversible, thereby suggesting that laparotomy is needed in cases of severe small bowel stenosis caused by intestinal anisakiosis, even if a long period of conservative treatment for the intestinal anisakiosis allowed the patient to pass successfully through the acute phase.

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Key words: Anisakiosis; Small intestine

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INTRODUCTION

Gastrointestinal anisakiosis, caused by an infection through the ingestion of *Anisakis sp.* larvae in raw or insufficiently cooked fish, commonly occurs in the stomach. Small bowel anisakiosis is rarely seen^[1-10]. It has been reported that anisakiosis is usually a self-limiting disease process

cured by conservative management for 1-2 wk after the onset of the symptoms^[5,8,9]. We herein present two cases of small intestinal anisakiosis which were treated surgically after long period of conservative management. We also discuss whether patients with severe small bowel stenosis, who have successfully passed through the acute phase of intestinal anisakiosis, can be conservatively managed.

CASE REPORTS

Case 1

A 59-year-old male was admitted on January 30, 2001 to our hospital, with complaints of mild urticaria, left-sided abdominal pain, and nausea. Four days before admission, the patient had eaten mackerel. Upon admission, abdominal CT showed a mild dilatation of the small intestine with ascites; small bowel edema and thickening of the intestinal wall were not detected. Beginning the day after admission, the patient received conservative medical treatment for acute pancreatitis, a diagnosis based on the serum amylase level of 1666 IU/L (normal range: 130-400). No evidence of eosinophilia was present. Abdominal pain continued after normalization of the serum amylase level. The titer of serum IgG antibodies for *Anisakis sp.* was 2.30 (normal range < 1.5). An upper gastrointestinal (UGI) series by Gastrografin^R showed a narrow segment (20 cm in length) of the small intestine and revealed a dilatation on the oral side of the intestine (Figure 1). Kerckring's folds disappeared completely in the narrow segment. Laparotomy was performed on the 35th post-admission day because the stenosis of the small intestine had not improved at all. Hence, the affected segment of the ileum was resected that measured 50 cm in length. Histopathological examination of the resected specimens revealed ulceration with infiltration of inflammatory cells in all layers of the intestinal wall. The structure of the wall was completely destroyed (Figure 2). The postoperative course was uneventful.

Case 2

A 62-old-male was admitted on November 19, 2004 to our hospital, with complaints of upper abdominal pain, nausea, and vomiting. Four days before admission, the patient had eaten sashimi and salted salmon. Abdominal CT demonstrated a remarkable thickening of the jejunal wall with ascites (Figure 3). An upper gastrointestinal series showed a narrow segment of the jejunum (20 cm in length) and revealed a dilatation on the oral side of the intestine (Figure 4). Kerckring's folds were visible. The titer of serum IgE-specific antibodies for *Anisakis sp.* was



Figure 1 UGI series showing a narrow segment of the small intestine with the disappearance of Kerckring's folds.

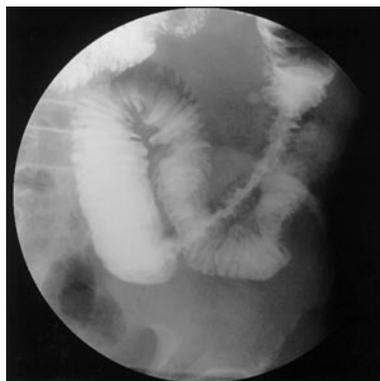


Figure 4 UGI series showing a narrow segment of the small intestine with the preservation of Kerckring's folds.

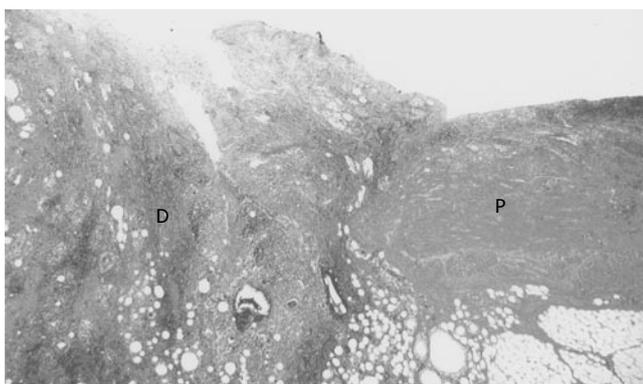


Figure 2 Resected specimens revealed histologically a complete destruction of the jejunal wall (D). P: Preserved wall of the intestine with an infiltration of inflammatory cells.

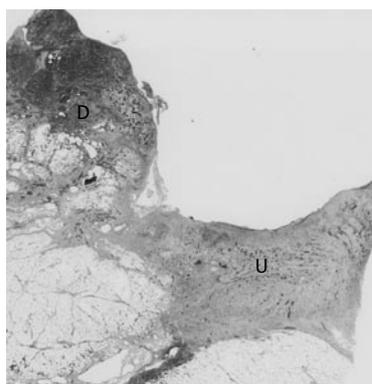


Figure 5 Resected specimens revealed histologically a perforated ulceration (U) with an infiltration of inflammatory cells. D: Destructive wall of the intestine.



Figure 3 CT showing a remarkable thickening of the intestinal wall.

14.3 (normal range < 0.34). Laparotomy was performed on the 23rd post-admission day because conservative therapy had not been effective. The affected segment of the jejunum was resected that measured 80 cm in length. Histopathological examination of the resected specimens revealed a perforated ulceration with infiltration of inflammatory cells in all layers of the intestinal wall and the formation of granulation tissue. The structure of the intestinal wall was completely destroyed, similar to Case 1 (Figure 5). The postoperative course was uneventful.

DISCUSSION

Anisakiosis, previously called anisakiasis, is the formal

terminology for the disease associated only with the genus *Anisakis*^[11]. In Japan, where raw fish is traditionally eaten in the diet, anisakiosis has been a public health problem^[12]. In Japan, most commonly reported anisakiosis is gastric anisakiosis. By contrast, in Italy and Spain, it has been reported that the disease has manifested as lesions in the small intestine in the majority of cases, but we are aware that the number of reported cases in these two countries does not reflect the frequency of anisakiosis there^[13,14].

In our diagnosis of gastrointestinal anisakiosis for the patients who manifested acute abdominal pain, raw fish ingestion prior to their admission was the most important aspect of their clinical history. Our patients had a history of ingesting raw fish. A positive result on a serological test has been shown to be helpful in diagnosis^[15]. In addition, imaging modalities, such as US and CT, have been reported to be useful^[2,8]. Typical CT findings include a relatively long segment of the symmetric wall thickening with luminal narrowing and diffuse contrast enhancement on the involved segment^[8]. A long segmental stenosis found in UGI series of our cases was similar to the CT findings.

For surgeons, small bowel stenosis would be one of the abdominal complications in patients with intestinal anisakiosis. Some reports argue that an early surgical approach should be avoided in cases where a small bowel obstruction suggests the possibility of intestinal anisakiosis^[8,13]. We would like to consider whether severe stenosis of the small intestine can be conservatively managed despite the fact that anisakiosis is reported to be a self-limiting disease. In our two patients, surgical intervention was eventually performed on the 23rd and 35th post-admission days, respectively. To our knowledge, there have been no reports of a long period of conservative

treatment of intestinal anisakiasis with severe small bowel stenosis in patients who successfully passed through the acute phase. When the intestinal wall has been completely damaged by the inflammatory reaction of anisakiasis, the change is irreversible. Our results indicate that laparotomy should be recommended for patients with severe long segmental stenosis caused by intestinal anisakiasis when the condition does not improve within 1-2 wk after conservative treatment.

In conclusion, surgeons should consider early laparotomy in the cases of patients with severe intestinal stenosis resulting from anisakiasis.

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An unusual cause of dyspnoea complicating right upper abdominal swelling

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Abstract

A middle aged, non-addict male presented with right upper abdominal pain and swelling with respiratory distress. Examination revealed central cyanosis, bipedal pitting edema with prominent epigastric and back veins. Liver was enlarged, tender, spanned 20 cm without any splenomegaly or ascites. Other systems were clinically normal. Laboratory investigations showed polymorphonuclear leucocytosis with slightly deranged liver function. Abdominal ultrasonography showed an abscess in the right lobe of the liver with compressed inferior vena cava (IVC), middle and left hepatic veins. Arterial blood gas (ABG) documented hypoxia with orthodeoxia and air-contrast echocardiography was suggestive of an intrapulmonary shunt. A diagnosis of hepato-pulmonary syndrome (HPS) was made with near normal liver function secondary to amoebic liver abscess. It reversed completely following successful treatment of the liver abscess.

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Key words: A-a O₂ gradient; Air contrast echocardiography; Hepato-pulmonary syndrome; Orthodeoxia; Amoebic liver abscess

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INTRODUCTION

Hepato-pulmonary syndrome (HPS) is a dreaded

complication of liver disease. It consists of the triad of hepatic dysfunction and/or portal hypertension, intrapulmonary vascular dilatation, and gas exchange abnormalities (PaO₂ < 70 mmHg or alveolo-arterial gradient > 20 mmHg). HPS occurs mostly in cirrhotic patients but it has also been reported in cases of non-cirrhotic portal hypertension with minimal hepatic dysfunction. Liver transplantation is the only therapeutic option of proven benefit. We present a case where a large amoebic liver abscess gave rise to transient portal hypertension due to Budd-Chiari Syndrome (BCS). The hemodynamic alteration led to development of HPS with minimal hepatic dysfunction and it reversed completely after successful treatment of the abscess.

CASE REPORT

A 38-year old non-addict male presented with right upper abdominal pain and progressively increasing right upper quadrant swelling for the last 3 mo. Along with it he also noticed swelling of both feet for 2 mo and respiratory distress for last 7 d. The abdominal pain was dull aching in character. There was no definite history of radiation or any aggravating or relieving factors. The patient denied any history of nausea, vomiting, and altered bladder or bowel habits. The bipedal edema was not associated with any facial puffiness, palpitation, chest pain or oliguria. His respiratory distress was progressively increasing and was not relieved on upright posture. He also noticed yellowish discoloration of his eyes and urine along with occasional low grade fever and weight loss. His past, personal and family history was non-contributory.

General examination revealed mild pallor, icterus, and bipedal pitting edema along with the presence of central cyanosis. The patient was febrile (oral temperature 39.2°C) with a respiratory rate of 28/min. The umbilicus was central, inverted with a single prominent epigastric and back vein. The venous flow was below upwards. Liver was enlarged, firm and tender with a sharp margin and smooth surface. The hepatic span was 20 cm and there was no audible bruit or rub. He did not have splenomegaly or ascites. The chest was dull on percussion below 4th intercostal space (ICS) along midclavicular line (MCL), below 6th ICS along midaxillary line (MAL) and below 8th ICS along infrascapular region on the right side, without any mediastinal shift. Auscultation revealed diminished breath sounds with diminished vocal resonance over the above region without any added sounds.



Figure 1 Chest X-ray showing elevated right dome of diaphragm.

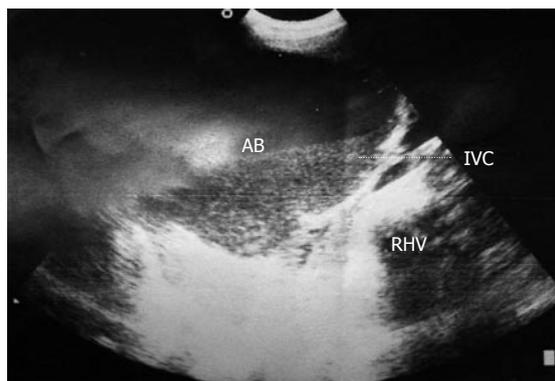


Figure 2 Abscess in right lobe of liver with compressed IVC and non-visualized middle and left hepatic veins.

Examination of the other systems was within normal limits.

A complete blood count showed hemoglobin 11.5 mg% and total leucocyte count of 11 200/cmm with polymorphonuclear leucocytosis. The liver function test revealed bilirubin of 4.5 mg/dL (direct: 3.1 mg/dL), AST: 65 U/L, ALT: 70 U/L, ALP: 350 U/L, protein: 7.1 g/dL (Alb: 3.7 mg/dL), P-time: 15.8 s (control 13 s); other biochemical parameters were unremarkable. Chest X-ray was normal except for an elevated right dome of diaphragm (Figure 1). Ultrasonography of the abdomen unmasked an abscess in right lobe of liver (13 cm × 14 cm), with mild ascites. The portal vein was 15.5 mm with normal splenic size. The IVC was compressed and the middle and left hepatic veins were not visualized (Figure 2). Doppler study showed absent phasic variation in above veins.

The viral markers were negative and an upper GI endoscopy was unrevealing. Bacterial culture of the anchovy sauce pus on CT guided aspiration was negative.

Arterial blood gas analysis was performed with the patient both in supine position and after standing upright for 5 min. It showed hypoxemia with orthodeoxia, along with an increased alveolo-arterial gradient [Supine PaO₂: 70 mmHg; Standing PaO₂ (after 5 min): 59 mmHg; Standing A-a O₂ gradient: 46 mmHg]. The hypoxia was partially corrected on 100% oxygen inhalation.

We performed an air contrast echocardiography. Ten milliliters of normal saline were agitated in two 10-mL syringes connected through a 3-way stopcock, and it was then injected through a 20 gauge right superior extremity intravenous line. Micro bubbles were detected immediately in the right sided cardiac chambers on apical 4-chamber view of 2-dimensional transthoracic echocardiography. Delayed visualization of micro bubbles in the left heart chamber (i.e., after 3 cardiac contractions following visualization of micro bubbles in the right heart chamber) was suggestive of intrapulmonary “right to left” shunting. Normally no bubbles should be detected in the left heart chambers even after 3 injections of agitated normal saline. If it is visible within 3 cardiac contractions it suggests intracardiac shunting. In our case micro bubbles were visible in the left heart chambers after 5 cardiac contractions. Chest radiography, electrocardiography, and echocardiography were performed to exclude primary pulmonary or cardiac pathology.

Hence, we diagnosed it as a case of HPS occurring as a rare complication of amoebic liver abscess due to transient portal hypertension with near normal liver function. The patient was put on tube drainage with intravenous metronidazole. The pig-tail catheter was removed after the drainage volume decreased and the IVC was fully decompressed. A repeat ABG after 8 wk showed normalization of both hypoxia and orthodeoxia (Supine PaO₂: 94 mmHg, Standing PaO₂: 91 mmHg, Standing A-a O₂ gradient: 9 mmHg).

DISCUSSION

Amoebic liver abscess is common in this part of the world. Development of BCS due to amoebic liver abscess though uncommon, has been previously reported^[1,2]. HPS is a specific entity identified in the recent past. Although many data have emerged, questions remain unanswered regarding its natural history and pathogenesis. Hepatic dysfunction appears to be the usual setting in which it occurs. Prevalence of HPS in cirrhotic patients ranges from 5% to 29%^[3,4]. However, there have been anecdotal reports of HPS in patients with portal hypertension, without evidence of hepatic dysfunction (e.g., congenital hepatic fibrosis^[5], nodular hyperplasia^[6], Budd-Chiari syndrome^[7]). HPS also has been reported in cases of non-cirrhotic portal hypertension^[8], where hepatocellular dysfunction is minimal.

In our case hepatic dysfunction was minimal but transient portal hypertension was evidenced by prominent epigastric vein and ultrasonographic documentation of dilated portal vein (15.5 mm). Development of HPS in this short period (3 mo) is unusual but has been reported^[2]. Portal hypertension plays an important role in the pathogenesis of HPS, probably by increased NO production^[9]. Hemodynamic alteration in BCS leads to increased sinusoidal pressure and increased shear stress. The activation of NO release, by shear stress, from endothelial cells through a specific G protein-mediated signaling pathway may be an important factor^[10]. HPS can be reversed by cavoplasty in cases of BCS due to inferior vena cava obstruction^[2]. In our case decompression of IVC by drainage of the abscess led to the reversal of HPS. Thus, the above mentioned mechanisms might form the

basis of development of intrapulmonary vascular dilatation as a result of elevated portal pressure, irrespective of the associated hepatic functional status.

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Digestive Disease Week
107th Annual of AGA, The American Gastroenterology Association
20-25 May 2006
Loas Angeles Convernction Center, California

American College of Gastroenterology Annual Scientific
20-25 October 2006
Las Vegas, NV

14th United European Gastroenterology Week, UEGW
21-25 October 2006
Berlin, Germany

APDW 2006: Asian Pacific Digestive Week 2006
26-29 November 2006
Lahug Cebu City, Philippines

EVENTS AND MEETINGS IN THE UPCOMING 6 MONTHS

Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
24-25 March 2006
Sydney - NSW
Falk Foundation e.V.
symposia@falkfoundation.de

10th International Congress of Obesity
3-8 September 2006
Sydney
Event Planners Australia
enquiries@ico2006.com
www.ico2006.com

Easl 2006 - the 41st annual
26-30 April 2006
Vienna, Austria
Kenes International

Prague hepatology 2006
14-16 September 2006
Prague
Foundation of the Czech Society of Hepatology
veronika.revicka@congressprague.cz
www.czech-hepatology.cz/phm2006

12th International Symposium on Viral Hepatitis and Liver Disease
1-5 July 2006
Paris
MCI France
isvhld2006@mci-group.com
www.isvhld2006.com

Falk Symposium 152: Intestinal Disease Part I, Endoscopy 2006 - Update and Live Demonstration
4-5 May 2006
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Falk Symposium 153: Intestinal Disease Part II, Immunoregulation in Inflammatory Bowel Disease - Current Understanding and Innovation
6-7 May 2006
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ILTS 12th Annual International Congress
3-6 May 2006
Milan
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www.its.org

Internal Medicine: Gastroenterology
22 July 2006-1 August 2006
Amsterdam
Continuing Education Inc
jbarnhart@continuingeducation.net

6th Annual Gastroenterology And Hepatology
15-18 March 2006
Rio Grande
Office of Continuing Medical Education
cmenet@jhmi.edu
www.hopkinscme.net

World Congress on Gastrointestinal Cancer
28 June 2006-1 July 2006
Barcelona, Spain
c.chase@imedex.com

International Conference on Surgical Infections, ICSI2006
6-8 September 2006
Stockholm
European Society of Clinical Microbiology and Infectious Diseases
icsi2006@stocn.se
www.icsi2006.se/9/23312.asp

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3-7 September 2006
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26-29 April 2006
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www.sages.org

Digestive Disease Week 2006
20-25 May 2006
Los Angeles
www.ddw.org

Annual Postgraduate Course
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Los Angeles, CA
American Society of Gastrointestinal Endoscopy
www.asge.org/education

American Society of Colon and Rectal Surgeons
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Gut hormones, and short bowel syndrome: The enigmatic role of glucagon-like peptide-2 in the regulation of intestinal adaptation

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primarily examines the physiology, mechanism of action, and utility of GLP-2 in the regulation of intestinal mucosal growth.

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Abstract

Short bowel syndrome (SBS) refers to the malabsorption of nutrients, water, and essential vitamins as a result of disease or surgical removal of parts of the small intestine. The most common reasons for removing part of the small intestine are due to surgical intervention for the treatment of either Crohn's disease or necrotizing enterocolitis. Intestinal adaptation following resection may take weeks to months to be achieved, thus nutritional support requires a variety of therapeutic measures, which include parenteral nutrition. Improper nutrition management can leave the SBS patient malnourished and/or dehydrated, which can be life threatening. The development of therapeutic strategies that reduce both the complications and medical costs associated with SBS/long-term parenteral nutrition while enhancing the intestinal adaptive response would be valuable.

Currently, therapeutic options available for the treatment of SBS are limited. There are many potential stimulators of intestinal adaptation including peptide hormones, growth factors, and neuronally-derived components. Glucagon-like peptide-2 (GLP-2) is one potential treatment for gastrointestinal disorders associated with insufficient mucosal function. A significant body of evidence demonstrates that GLP-2 is a trophic hormone that plays an important role in controlling intestinal adaptation. Recent data from clinical trials demonstrate that GLP-2 is safe, well-tolerated, and promotes intestinal growth in SBS patients. However, the mechanism of action and the localization of the glucagon-like peptide-2 receptor (GLP-2R) remains an enigma. This review summarizes the role of a number of mucosal-derived factors that might be involved with intestinal adaptation processes; however, this discussion

INTRODUCTION

Advances in surgical and medical care have made it possible for those with massive small bowel resections to survive. Unfortunately, a loss of greater than 50% of the small intestine often results in short bowel syndrome (SBS)^[1-4]. SBS is characterized by the inefficient absorption of nutrients and fluids resulting in severe diarrhea, dehydration, electrolyte imbalances, nutrient deficiencies, weight loss, and frequently, a long-term dependence on parenteral nutrition^[4-6]. In the pediatric population, total parenteral nutrition (TPN) maintenance-associated complications frequently result in sepsis, secondary liver failure, and high morbidity^[5,7,8]. The development of therapeutic strategies that could reduce the consequences of SBS and long-term parenteral nutrition while enhancing the intestinal adaptive response would be valuable.

Numerous non-nutritional factors are potential stimulators of intestinal adaptation including peptide hormones, growth factors, and neuronally-derived components^[9]. Some of the growth factors that have been shown to stimulate intestinal growth include glucagon-like peptide-2 (GLP-2), epidermal growth factor (EGF), growth hormone (GH), insulin-like growth factor-1 (IGF-1), neurotensin (NT), intestinal trefoil factor (ITF), transforming growth factor (TGF- α ; TGF- β), and

Table 1 Growth factors as potential stimulators of intestinal adaptation

Growth factor	Amino acids /MW	Mucosal growth factors and SBS	
		Main source	GI receptor localization
GLP-2	33 aa/3.8 kDa	Ileal and colonic EE L-cells, pancreatic α cells	GLP-2R; EE cells, myenteric/submucosal neurons of ENS
EGF	53 aa/6.4 kDa	Submandibular gland, duodenal Brunner's gland, epithelial and Paneth cells	EGFR/HER/ErbB, HER2/Neu/ErbB2, HER3/ErbB3, HER4/ErbB4
KGF (FGF-7)	194 aa/2.5 kDa	Fibroblast-like stromal cells in epithelial tissue	KGFR; a/bFGFR epithelial cells
Hb-EGF	86 aa/22 kDa	Smooth muscle cells	Activate all as listed for the EGFR
GH	191 aa/22 kDa	Liver	CRF type-1, PRL-R
TGF- α	50-53 aa/6.2-6.4 kDa	Epithelial cells	Activate all as listed for the EGFR
NT	13 aa/?	Ileum/colon >> proximal SB, EE N-cells	NTR1
PYY	36 aa/42 kDa	Ileal and colonic EE L-cells	Y1-R; EE cells, myenteric/submucosal neurons of ENS
GRP	14 aa/?	Neuroendocrine cells in the ENS, fibroblasts	GRPR antrum, upper GI tract
CCK	8, 33 aa/39 kDa	Duodenal EE I-cells	CCK α >> CCK β upper GI tissue myenteric/submucosal neurons of ENS
Gastrin	17 aa/21 kDa	Gastric antrum G-cells	CCK β >> CCK α
	34 aa/40 kDa	Pancreatic D-cells	upper GI tissue
IGF-I/II	I-70 aa/7.6 kDa,	Proximal SB >> distal, liver,	IGF-IR and IGF-IIR
	II-67 aa/7.4 kDa	Fibroblasts	epithelial cells, glial cells, fibroblasts

Many of these factors include peptide hormones, growth factors, and neurovascular components. SB, small bowel; ENS, enteric nervous system; EE, enteroendocrine; GLP-2, glucagon-like peptide-2; EGF, epidermal growth factor; Hb-EGF, heparin-binding EGF; KGF, keratinocyte growth factor (FGF-7); FGF, fibroblast growth factor; GH, growth hormone; CRF-1, cytokine-related family type-1 (JAK-STAT pathway); PRL, prolactin (subgroup of the CRF-1 superfamily); TGF- α , transforming growth factor- α ; NT, neurotensin; PYY, peptide tyrosine tyrosine; GRP, gastrin-releasing peptide; CCK, cholecystokinin; IGF-I/II, insulin-like growth factor I/II.

fibroblast growth factor (FGF)^[10]. Table 1 lists some of the mucosa-derived humoral factors that have been shown to promote intestinal adaptation following intestinal resection.

GLP-2 is a potential therapy currently under consideration for the treatment of gastrointestinal disorders that are associated with insufficient mucosal function. Evidence from both animal studies and clinical trials demonstrate that GLP-2 is a trophic hormone that plays an important role in the regulation of intestinal adaptation^[11]. Under normal conditions, GLP-2 is secreted into the circulation in response to the ingestion of a mixed meal^[12-15], particularly following one that contains carbohydrates, fatty acids, and fiber^[16-18]. These studies have shown that following nutrient-induced release or exogenous administration, GLP-2 is involved in the regulation of cell proliferation, apoptosis, nutrient absorption, motility, as well as epithelial and intestinal permeability^[15,19-25].

Initial pilot Phase II trials in SBS patients treated with teduglutide, a GLP-2 analog, observed that there were modest increases in fluid and nutrient absorption. Based upon the positive results of this trial, Phase III studies in adults with SBS were initiated. Subsequently, a recent article in *GUT* shows that teduglutide, using various dosing modalities over a 21 d experimental period, was safe, well tolerated, and promoted intestinal growth in SBS patients having either an end jejunostomy or a colon in continuity^[26]. Moreover, teduglutide therapy in these SBS patients resulted in a significant increase in small intestinal villus height, crypt depth, and mitotic index. The authors state that the most significant clinical benefits of teduglutide in treating SBS patients were the reduction of intestinal wet weight excretion and in improving wet

weight absorption^[26]. There are several other clinical trials underway that are examining potential therapeutics for the treatment of SBS, unfortunately, Zorbitive[®] (hGH, glutamine) remains the only option presently available for prescriptive use.

This review summarizes the current understanding of a number of growth factors and their role in the treatment of SBS. As it is unlikely that there is just one specific growth factor responsible for maintaining mucosal growth, an important consideration is that there is likely some degree of regulation, transactivation, and crosstalk, between established stimulators of mucosal growth (i.e. epidermal growth factor, insulin-like growth factor-I, polyamines, GLP-2). Thus an understanding of the collective influence of these promoters of intestinal adaptation should not be dismissed. However, the primary focus of this discussion is to examine the physiology, mechanism of action, and utility of GLP-2 in the regulation of intestinal mucosal growth.

Discovery of GLP-2

The first indication that GLP-2 was trophic to the bowel was the observation that patients with glucagonomas had significantly elevated circulating enteroglucagon levels that was secondarily associated with intestinal mucosal hyperplasia^[27,28]. The correlations between serum enteroglucagon levels and mucosal growth of the small intestine provided further evidence for the trophic effects of GLP-2^[27]. A major breakthrough came in 1996 when it was shown that injection of proglucagon-producing tumors into nude mice resulted in significant mucosal hyperplasia^[29]. Isolation of the various components of these proglucagon-producing tumors revealed that GLP-2 was the trophic factor responsible for the increased

mucosal growth.

Additional evidence for intestinotrophic properties of GLP-2 came from studies in rats and mice in which GLP-2 administration induced intestinal hypertrophy i.e., significant increases in protein and DNA content, villus height, and bowel weight^[21,25,30,31]. Moreover, GLP-2 treatment reduced mucosal atrophy associated with TPN administration and augmented the adaptive growth response following massive small bowel resection^[19-21,32]. Our studies showed that in parenterally maintained animals (no luminal nutrition), infusion with GLP-2 stimulated intestinal adaptation following small bowel resection^[18]. Without the infusion of GLP-2, intestinal adaptation did not occur. Similarly, the immunoneutralization of endogenous circulatory GLP-2 reduced the intestinal post-resection adaptive response in rats and rabbits^[33].

What is GLP-2?

GLP-2 is a member of the PACAP (Pituitary adenylate cyclase activating peptide)/Glucagon superfamily that includes the enteroglucagon/proglucagon-derived peptides (PGDP) that are produced in the L-cells of the small intestinal and colonic mucosa. The L-cells have been identified as the cellular origin of enteroglucagon (Figure 1)^[24,34,35]. In 1983, the full sequence of hamster proglucagon, and later that year, the human sequence, were published revealing the sequences of GLP-1 and GLP-2^[36,37]. GLP-2 is described as glucagon-like as it shares approximately 50% sequence homology with glucagon^[37]. Sequencing of GLP-2 from human and pig intestine has confirmed the structure of GLP-2 as a 33 amino acid peptide that contains alanine at position 2 of the N-terminus (making it easily degraded by exopeptidases) and ends with a carboxy-terminal Asp residue^[45]. GLP-2 and the other PGDPs are derived following the proteolytic cleavage and other enzymatic modifications of proglucagon in the organelles of the pancreatic α -cells and intestinal L-cells^[38,39]. Interestingly, GLP-2 and the other products of post-translational processing (glicentin, GLP-1, oxyntomodulin) are produced in the intestinal L-cells, while glucagon and the major proglucagon fragment (MPGF) are produced in the α -cells of the pancreas^[40]. They are formed from the same precursor molecule and therefore must be secreted in parallel. Differential expression of prohormone convertase (PC), the enzyme responsible for cleaving proglucagon into various hormonal products in the different cells, accounts for the different products. The PC2 enzyme, which has a greater abundance in the pancreatic α -cells, preferentially produces glucagon as its end product^[41-43]. On the other hand, the PC1/3 enzyme, which is found in greater abundance in the intestinal L-cell, predominantly generates the glucagon-like peptides (GLP-1/2)^[41]. Therefore, the differential processing of proglucagon in pancreatic α -cells by PC2, and by PC1/3 in the intestinal L-cell, might generate two unique hormonal products from the same precursor with each having a different physiological effect^[43].

Initially, the GLP-2 sequence was identified in all mammalian cDNA, but not in other species. This led researchers to theorize that GLP-2 was a late evolutionary addition. Subsequent studies have demonstrated that fish,

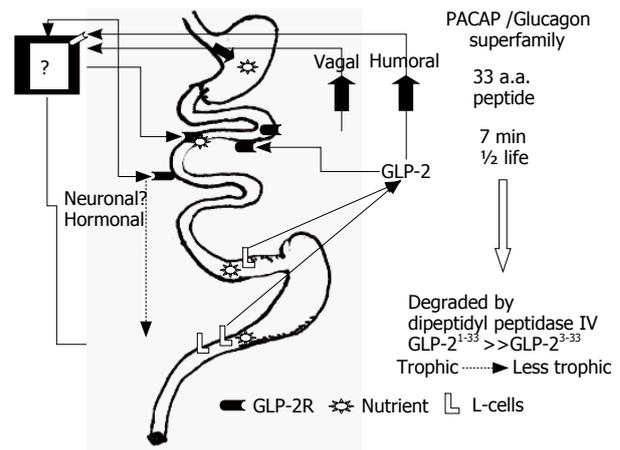


Figure 1 Potential pathways involved in the production, release, activity, and sites of action of GLP-2. GLP-2 produced by the L-cell is induced following the ingestion of a meal and can be induced either by direct stimulation of the L-cell, or potentially following the release of upstream neuronal /hormonal agonists. The GLP-2R has been localized to components of the enteric nervous system (ENS) and to regions of the brain, thus the growth-promoting effects associated with GLP-2 may involve interactions with the brain-gut axis. Potentially the ENS / vagal interface might be responsible for the early release of GLP-2.

chickens, and lizards generate GLP-2 in the gut as a result of tissue-specific alternative RNA splicing of proglucagon RNA transcript^[44].

Site of GLP-2 production

The distal regions of the small bowel and/or the large intestine are necessary for GLP-2 production. This was demonstrated in a study in which patients with SBS, without a colon in continuity, had significant reductions in their post-prandial circulatory GLP-2. Subjects that had 140 cm or less of small bowel, with colon in continuity, exhibited a significant increase in their serum GLP-2 levels^[46,47]. Conversely, fasting and post-prandial serum GLP-2 levels were elevated in SBS patients (colon intact, no ileum) compared with sex- and age-matched controls^[46]. The locale of the GLP-2-containing tissue in the human intestine is debatable, though it was recently reported that the number of immunoreactive GLP-2 cells increase in a proximal to distal distribution in normal colonic tissue^[48].

However, there is debate as to the contribution of different regions of the bowel to total GLP-2 production. A 90% intestinal resection, leaving only 10 cm of distal ileum, results in a significant elevation in serum GLP-2 levels in the rat^[15]. These elevations in circulatory GLP-2 correlate with several measurable parameters of intestinal adaptation^[18]. As the remainder of the small bowel had been removed, this suggests that the distal ileum and/or the colon are the major sites of the L-cells, the cells responsible for the production of GLP-2 (Figure 1). Others describe that in the rat, TNBS (trinitrobenzene sulphonic acid) -induced ileitis resulted in no change in the number of GLP-2 immunoreactive cells in the ileum, yet colonic GLP-2 immunoreactivity was increased (personal communication, Jennifer O'Hara and Dr. K.A. Sharkey).

Interestingly, in a rat SBS model in which only 20 cm of jejunum was anastomosed directly to the ascending colon^[49] (i.e., removal of 80% of the distal small bowel

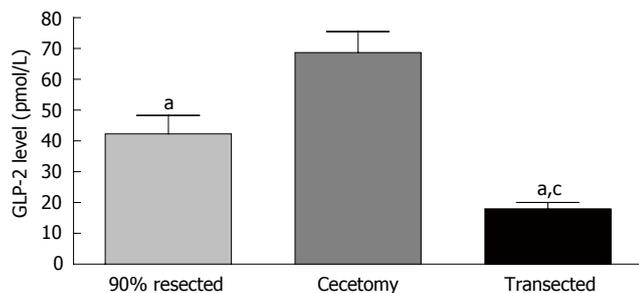


Figure 2 An experimental rat model of SBS-induces changes in circulatory GLP-2 levels. The distal regions of the small bowel and/or the large intestine are necessary for GLP-2 production. In these experiments, the entire proximal small bowel was removed; therefore the distal ileum and/or the colon are likely the cells responsible for the production of GLP-2 as all other bowel tissue had been removed. Resected = 90% intestinal resection leaving a 10 cm ileal remnant. CEC = cecetomy + removal of all but 20 cm of jejunum. The remnant jejunum was anastomosed to the ascending colon. ^a*P* < 0.05 vs cecetomized animals; ^c*P* < 0.05 vs 90% proximal resected animals that were enterally-fed (10 cm remnant ileum).

including the ileum and cecum), circulatory GLP-2 levels were significantly increased in comparison to both controls and the 90% resection animals with ileum intact (Figure 2). This strongly suggests that the colon is an important source of GLP-2. These findings emphasize the importance of determining if the increase in serum GLP-2 levels are the result of either an increase in the L-cell population, or alternatively, as a consequence of augmented GLP-2 production by the L-cells. Our ongoing experiments suggest, at least in this rat model of SBS, that increased GLP-2 production is the result of an increase in the colonic L-cell population (unpublished observations).

GLP-2 receptor and activity

Currently, the localization of the glucagon-like-2 receptor (GLP-2R) remains an enigma. Conflicting data in the literature report the GLP-2R to be located on enteroendocrine cells^[50], on non-epithelial elements such as neuronal cells^[51], and subepithelial myofibroblasts (Figure 1)^[52]. Moreover, the localization of the GLP-2R in the bowel might be region-specific. In a recent study by Orskov *et al*, GLP-2 receptors were found throughout the small and large bowel in humans, mice, marmoset, and rat^[52]. Furthermore, the intestinal region that had the greatest GLP-2R immunoreactivity was the proximal small bowel. These data were confirmed by both immunohistochemistry and *in situ* hybridization that showed that the GLP-2R was localized to cells lying beneath the basal membrane of enterocytes^[52].

The specific GLP-2 receptor has been isolated, sequenced, and found to be expressed in both the gut and in the hypothalamus (Figure 1)^[53]. GLP-2 binds to a 7 transmembrane (7-TM) G protein-coupled receptor that is comprised of 550 amino acids. In humans, the expression of the GLP-2R is localized to specific enteroendocrine cells in the stomach as well as the small and large bowel^[53-55]. Currently there have been only two human cervical carcinoma cell lines, including HeLa cells, in which GLP-2R mRNA transcripts have been detected^[56]: the majority of the signal pathway determinations have been carried out in cells transfected with the GLP-2R^[55]. The

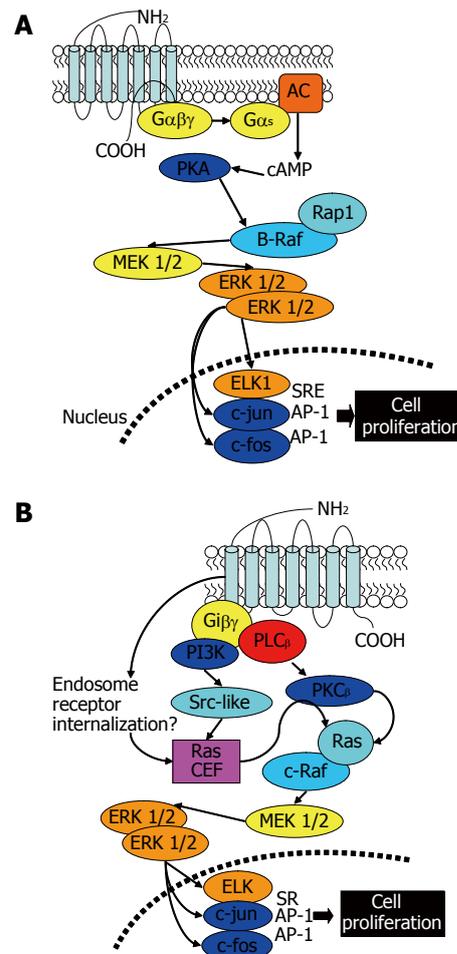


Figure 3 A: GLP-2 stimulation of the G_s-Coupled Receptor induced cell proliferation. The proposed GLP-2 /GLP-2R signaling pathway as characterized in cells that were transfected with the GLP-2R. See section on GLP-2 receptor-activity for description (pages 11-12); B: GLP-2 stimulation of the G_α-Coupled Receptor induced cell proliferation. Possible trafficking pathways triggered by GLP-2 in cells that tested negative for the characterized GLP-2R. Potentially, GLP-2 induces divergent signaling pathways which results in an increase in the rate of cell proliferation. See section on GLP-2 receptor-activity for description (pages 11-12).

enteroendocrine cells that do express the GLP-2R have also been found to express other gut hormones such as glucagon-like peptide-1, glucose dependent insulinotropic peptide, peptide YY, and serotonin^[50]. PACAP and VIP share the same VPAC₁ receptor with equal affinity^[54], thus it might be possible that other members of this family also share receptors.

There are a number of unanswered questions regarding the signaling mechanism involved in how GLP-2 stimulates mucosal growth. The initial studies on intracellular trafficking was done entirely in cell lines (BHK /COS) that had been transfected with the characterized GLP-2R^[55,57]. These data showed that GLP-2/GLP-2R signaling involved classic G protein-coupled G_αs activation of adenylyl cyclase (AC), increased cAMP and PKA accumulation, and eventually ELK-1/c-fos/c-jun gene activation and increased cell proliferation (Figure 3A). Conversely, in epithelial cell lines devoid of the GLP-2R (as confirmed by both Western blot analysis and by the absence of mRNA transcripts), GLP-2 administration was still capable

of increasing cell proliferation^[56,58,59]. Thymidine uptake as a measure of an increased proliferation was significantly inhibited following pre-incubation with pertussis toxin ($G\alpha_i$ inhibitor) or MAPK blockade, implying that $G\alpha_i$ or EGFR/TyrK trafficking pathways are involved (Figure 3B). These studies also revealed that, in comparison with the transfected cell lines in which increases in cAMP correlated with augmented growth, there was a significant correlation between increased cell proliferation and decreased cAMP accumulation.

Furthermore, the possibility that there are multiple signaling mechanisms for GLP-2 is suggested by recent observations regarding the divergent effects of GLP-2 administration on epithelial cell proliferation. These effects were dependent on both the cell line and the dose of GLP-2 administered. Two independent studies demonstrated that administration of GLP-2 results in the inhibition of proliferation of non-transformed small intestinal epithelial cell lines (IEC-6, IEC-18), while increasing proliferation of cancer-derived human colonic epithelial cell lines (Caco-2, Colo 320)^[59,60]. Moreover, there can be divergent effects of GLP-2 on epithelial cell migration, as GLP-2 significantly increased migration in the small intestine-derived IEC-6/IEC-18 cells, but had no effect on epithelial cell migration in the colonic Caco-2 or the Colo 320 cells^[60].

An article recently published in *Science* may have uncovered another important component of the GLP-2 signaling pathway^[61]. It is established that the intestinal epithelium undergoes constant renewal along the crypt-villus axis and that the β -catenin/T cell factor (TCF) transduction pathway is involved in these proliferation/differentiation events^[62-64]. These data reveal that GLP-2 treatment of mice increases β -catenin levels suggesting that β -catenin might be a downstream mediator of GLP-2-induced crypt cell proliferation. In addition, the expression of a cDNA encoding human R-spondin1 (hRSpo1) in KI chimeras led to a substantial increase in the diameter, length, and weight of the small intestine, diffuse thickening of the mucosa, and crypt epithelial hyperplasia^[61]. Their data showed that the administration of hRSpo1 potently affects proliferation of the intestinal epithelium through activation of β -catenin; the activation of this pathway by hRSpo1 indicates that the effect might be directly stimulated by receptor-mediated binding. It is interesting that the expression of hRSpo1 in human intestinal enteroendocrine cells, consistent with the role of this protein as a crypt cell mitogen, is similarly localized to the expression of GLP-2^[61]. This is certainly an exciting find in regards to mapping out potential signaling pathways involved in GLP-2-induced intestinal growth.

Collectively, this dichotomy suggests that there are divergent signaling pathways involved in how GLP-2 stimulates cell proliferation. Secondly, it begs the question: are other uncharacterized GLP-2R's or transactivation signaling mechanisms involved in the intestinotrophic effect of GLP-2 other than that initially described by Munroe *et al.*^[53]?

GLP-2 interactions with the ENS/CNS

There is evidence that the GLP-2R is expressed on both

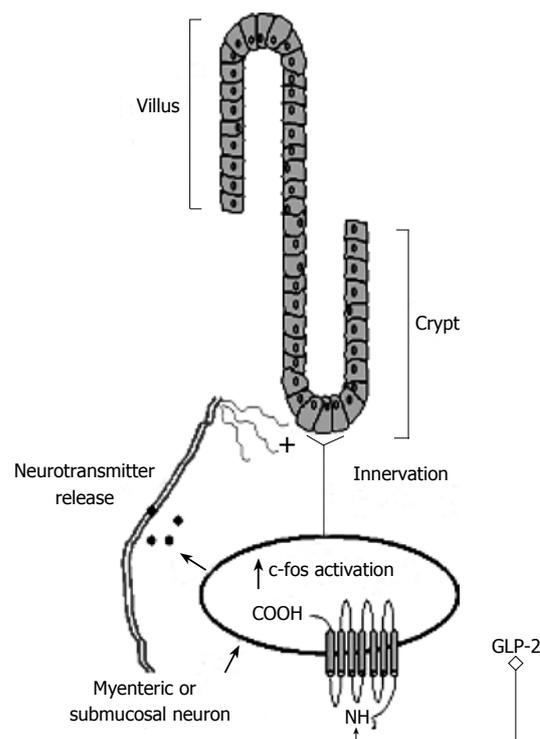


Figure 4 GLP-2 stimulation of the enteric nervous system. GLP-2 binds to 7-TM G protein-coupled receptors localized to components of the ENS. This results in a conformational change in the GLP-2R that eventually activates c-fos expression via the $G\alpha_s$, AC, c-AMP pathway. This suggests that a rise in circulatory GLP-2 levels might stimulate the GLP-2R leading to an increased stimulation of the myenteric and/or submucosal neurons. Potentially the trophic effects on the gut mucosa associated with GLP-2 are the result of a second downstream mediator, either a neurotransmitter or direct innervation that is activated upon GLP-2's initial stimulation of the ENS localized GLP-2R.

murine enteric neurons and cerebral cortex astrocytes^[51,65]. Bjerknes and Cheng showed by *in situ* hybridization that the GLP-2R is located on nonepithelial elements of the enteric nervous system. These results were further verified by reverse-transcriptase polymerase chain reaction (RT-PCR) for the GLP-2R mRNA^[51]. Moreover, the GLP-2R was located on neurons and not on glial cells. These experiments demonstrate that GLP-2 is capable of inducing *c-Fos* activity in enteric neurons in as little as 7 min, and achieved maximal expression by 15 min. After 90 min, the increase in GLP-2-induced neuronal *c-Fos* activity was followed by increased *c-Fos* activity in the crypt cells of the jejunum and colon. These observations suggest that GLP-2 stimulates the GLP-2R leading to an increased *c-Fos* expression in myenteric neurons (Figure 4), which is secondarily followed by both an increase in *c-Fos* expression in the crypt cells and the stimulation of columnar crypt cell growth. Tetrodotoxin (TTX) pre-treatment abolished *c-Fos* expression in the crypt cells revealing that GLP-2 signaling in the bowel might be dependent on a transduction pathway requiring enteric neurons^[51]. Potentially the intestinotrophic property associated with GLP-2 involves a second downstream mediator that is released upon GLP-2's initial stimulation of the ENS (Figure 4).

The GLP-2R is also localized to regions of the hindbrain^[66]. The dorsal medial hypothalamus (DMH) contains a dense plexus of GLP-2 immunoreactive fibers that express GLP-2 receptor mRNA (Figure 1). Though

research regarding GLP-2's involvement in neuronal activation is still in its infancy, it is interesting that GLP-2's effects on enteric /vagal pathways are remarkably similar to those characterized for cholecystokinin^[67,68]. This possibility has led to discussions regarding GLP-2 as a factor involved in the regulation of feeding. There is evidence that GLP-2 administration can inhibit feed intake^[66,69,70] however, other investigators report no effect on appetite or energy intake at physiological concentrations of GLP-2^[71]. This area of GLP-2 physiology requires further investigation.

These observations suggest a novel pathway of GLP-2 action as perhaps either a meal-stimulated endogenous release of GLP-2 from the L-cell, or potentially, the therapeutic administration of GLP-2, could activate enteric neuronal activity, resulting secondarily in the release of downstream mediators capable of inducing crypt cell proliferation (Figure 4). Currently, no studies have been published in which the expression of the GLP-2R in the human enteric nervous system was examined.

GLP-2 receptor antagonists

An obstacle when examining mechanistic pathways involved in GLP-2-induced small bowel adaptation is the lack of effective GLP-2R antagonists. One possibility would be to use the primary degradation product (GLP-2³⁻³³) to see if the interaction with the GLP-2R could reduce the adaptive response to intestinal resection. These results might be difficult to interpret as the treatment of mice with either GLP-2¹⁻³³ or GLP-2³⁻³³ induces significant growth in both the small and large bowel, though the growth response induced by the degradation product is reduced^[72]. Furthermore, functional studies and binding data indicate that GLP-2³⁻³³ acts as both a partial agonist and as a partial antagonist on the GLP-2R^[72]. However, there is recent data that demonstrates GLP-2³⁻³³ antagonizes the growth-promoting activities of GLP-2¹⁻³³ *in vitro* and *ex vivo*^[73]. Shin *et al* suggest that endogenous GLP-2¹⁻³³ regulates the intestinal growth-promoting response via modulation of crypt-cell proliferation and villus apoptosis^[73]. Collectively, these findings do not support the use of GLP-2³⁻³³ as a full antagonist of the GLP-2 receptor.

GLP-2 inactivation and metabolism

As mentioned earlier in this review, GLP-2¹⁻³³ contains the amino acid alanine at position 2 thereby making it susceptible to degradation by the exopeptidase dipeptidyl peptidase IV (DPP-IV)^[74]. For excellent reviews on the structural and functional properties of DPP-IV, see Mentlein or Lambier^[75,76]. The primary metabolism of GLP-2¹⁻³³ by DPP-IV, which cleaves off two N-terminal amino acids, results in the formation of GLP-2³⁻³³, a potential receptor antagonist /agonist^[72,73]. The elimination half-life of intact GLP-2¹⁻³³ in humans is 7 min, whereas that of cleaved GLP-2³⁻³³ is 27 min^[14], thus the cleavage of the NH₂ terminal may potentially disrupt the signal transduction pathway and reduce GLP-2's biological effectiveness. The administration of GLP-2 to DPP-IV deficient rats results in higher GLP-2¹⁻³³ serum levels which correlate with a significant increase in small intestinal weight^[74,77]. Similar increases in intestinal hypertrophy also

occur in rats and mice administered a synthetic DPP-IV resistant GLP-2 analog (ALX-0600)^[31,74] suggesting that the inactivation of GLP-2¹⁻³³ by DPP-IV is a crucial limiting factor regarding GLP-2's trophic effect on the gut mucosa. In humans, the replacement of alanine in position 2 of the GLP-2 peptide with glycine, has been shown to extend the half life from approximately 7 min, as for GLP-2, to 0.9-2.3 h.^[26] Thus, DPP-IV resistant GLP-2 analogues might be useful therapeutically to help improve mucosal regeneration.

In contrast, there is evidence that DPP-IV may not be the only factor important in the degradation of GLP-2^[78]. Geier *et al* did not detect an observable role for DPP-IV in the regulation of GLP-2 in DPP-IV^{-/-} mice, as there were no measurable differences in GLP-2 levels or plasma DPP-IV-like activity between DPP-IV^{+/+} and DPP-IV^{-/-} mice. Although DPP-IV^{-/-} mice lack the *DPP-IV* gene, they do possess the genes for FAP, DP8, and DP9, and might have sufficient dipeptidyl peptidase levels to regulate GLP-2 cleavage. Moreover, the mice lacking the *DPP-IV* gene do not have either intrinsic resistance, or an enhanced rate of repair, in DSS-induced colitis. The authors concluded that residual dipeptidyl peptidase levels in the DPP-IV^{-/-} mice results in insufficient GLP-2¹⁻³³ bioavailability to protect these mice from DSS (dextran sodium sulfate) -induced damage. The association between GLP-2 and DPP-IV family members will require further study.

Plasma concentrations of GLP-2 are not only influenced by the rate of secretion, but by the elimination or clearance rate. The kidney is important in the clearance of GLP-2 as there are increased circulatory levels in patients with renal failure^[79,80]. In addition, experimental nephrectomy results in delayed clearance and increased circulating levels of GLP-2 in rats^[81]. The increase in circulatory GLP-2 levels following nephrectomy is not surprising as it has been shown that the kidney is the site of greatest DPP-IV activity^[75].

GLP-2 and intestinal inflammation

The treatment of animals with GLP-2 reduces harmful symptoms of inflammation associated with colitis as well as the mortality and the severity of both indomethacin and TNBS-induced enteritis^[35,82,83]. Moreover, studies within our lab reveal that GLP-2 ameliorates inflammatory parameters associated with TNBS-induced ileitis (Gastroenterology abstract, T1530, 2005). These reductions in inflammation (decreased myeloperoxidase activity, interleukin-1 β , and inducible nitric oxide synthase protein in mucosal tissue) 5 d post-TNBS occurred whether the GLP-2 was given simultaneously with TNBS, or 2 d post-TNBS injection. Others have observed that GLP-2 treatment initiated following chemotherapy enhances intestinal recovery^[84]. A second report also showed the benefits of GLP-2 treatment as a preventative against cancer therapy-induced mucosal damage^[85]. Teduglutide, a GLP-2 analog, given daily prior to whole body gamma-irradiation significantly increased crypt stem cell survival in mice when compared with vehicle-treated controls, though the protective effect was only observed when teduglutide was given prior to irradiation^[85]. Collectively, these reports indicate that GLP-2 might be a useful therapeutic strategy in

the treatment of diseases associated with intestinal inflammation.

There are conflicting reports regarding the effect of intestinal inflammation on GLP-2 production, particularly on the site of GLP-2 production. Intuitively, any situation that depletes the GLP-2-producing L-cells could potentially reduce the ability of GLP-2 to repair the mucosa. This could be the case in Crohn's/colitis patients as inflammation of the intestine likely disrupts the L-cells. In a mouse-model of T-cell-induced inflammatory bowel disease (IBD), the amount of GLP-2 was significantly reduced in the colon^[86], however, others report that TNBS-induced ileitis results in an increase in colonic GLP-2 immunoreactivity. (O'Hara and Sharkey, personal communications). Regarding the latter, perhaps an increase in local and circulatory GLP-2 is the result of IBD-induced diarrhea, thus increasing the nutrient load in the distal bowel, a well-described potent stimulus for inducing GLP-2 production^[12,15,17]. Though speculative, perhaps increased growth and nutrient absorption in the proximal small intestine as a result of increased GLP-2 production would be successful in ameliorating inflammatory processes. Conversely, Schmidt *et al* found that both the fasting and meal-induced plasma levels of GLP-2 were not different between healthy controls and IBD patients^[87]. This report suggests that L-cell secretion of GLP-2 is not altered in IBD. The discrepancies between these observations could be the result of model/species difference, or site-specific effects of inflammation in the bowel.

While GLP-2 is the most prominent enterotrophic peptide, there are a number of hormones that might be involved in the regulation of mucosal growth. These are briefly discussed as it is likely that processes involved in intestinal adaptation are regulated by a complex array of hormones, neuropeptides, and cytokines. A number of mucosal growth factors that might be useful for the treatment of short bowel syndrome are listed in Table 1.

Insulin-like growth factor-I

Another growth factor that might have a potential role in enhancing mucosal growth is insulin-like growth factor-I (IGF-I). IGF-I is produced mainly in the liver, but it is also synthesized locally in the gut^[88]. There is evidence that IGF-I stimulates intestinal growth under experimental conditions of TPN therapy, intestinal resection, and radiation therapy^[89]. In addition, the over-expression of the gene encoding for IGF-I is associated with increased small bowel length, small bowel weight, and crypt cell proliferation^[90]. Conversely, others report that ileal IGF-I levels remain unchanged following intestinal resection^[90,91].

Most of the circulating IGF-I is bound to IGF-binding proteins (IGFBPs) that potentially protect IGF-I from degradation thereby modulating the activity of IGF-I^[92,93]. IGFBPs are capable of controlling the availability of circulating IGF-I to target tissues and are thus key components in the GH-IGF-I somatotrophic axis. In humans, six IGFBPs have been identified and shown to modulate IGF-I actions differently^[94]. Serum levels, jejunal tissue protein, and mRNA levels of IGF-I are reduced following small bowel resection^[95]. One possibility

is that there is a significant decrease in IGFBP-3 mRNA following intestinal resection, thus this rapid decrease in the IGFBP-3 could increase IGF-I bioavailability resulting in an enhanced adaptive response^[96].

In SBS rats maintained on parenteral nutrition, IGF-I infusions induced jejunal hyperplasia and normalized ion transport^[97]. Thus acute IGF-I treatments might prove to be beneficial in easing the transition from parental to enteral feeds thereby avoiding some of the long-term dilemmas associated with TPN therapy^[98]. However, there is no significant evidence of serum or tissue concentration fluctuation of IGF-I following intestinal resection, therefore its utility as a treatment for SBS remains unclear.

Epidermal growth factor

Most of the EGF family of peptides (transforming growth factor- α , amphiregulin, heparin-binding EGF, epiregulin, betacellulin, neuregulin, neuregulin-2) are trophic to the gastrointestinal tract as they both stimulate crypt cell proliferation and suppress apoptosis^[99]. There is evidence that EGF administration at the time of small bowel resection enhances the adaptive response. This appears to be accomplished by inducing mucosal hyperplasia and increasing nutrient absorption, while reducing intestinal permeability, weight loss, and apoptosis^[49,100,101]. It is important to note that EGF therapy is most effective for intestinal adaptation when it is administered early following resection^[102]. Very few studies have examined serum EGF levels following small bowel resection. Shin *et al* showed that small bowel resection does not change serum EGF levels, yet there was an increase in salivary and a decrease in urinary excretion of EGF^[103]. Ileal epidermal growth factor receptor (EGFR) levels were significantly increased suggesting that the increased salivary EGF (significant endogenous source of EGF), enhanced EGF-R expression, and reduced urinary excretion of EGF, leads to an increase in ileal utilization of EGF following resection. Compared with control mice, the removal of the submandibular glands significantly attenuated the increase in villus height, total protein, and DNA content of the small bowel following massive small bowel resection^[104].

It has also been suggested that intestinal adaptation following resection requires a functional EGFR^[105]. Selective inhibition of the EGFR with an orogastric EGFR inhibitor (ZD1839, 50 mg/kg per day) results in impaired intestinal adaptation after small bowel resection^[106,107]. There is also an impaired adaptive response to intestinal resection in waved-2 mice-mice that are predisposed to a naturally occurring mutation that results in a reduction in EGFR protein tyrosine kinase activity. *In vitro* studies demonstrate that serum from intestinal resection mice or rats is capable of stimulating a proliferative response in rat intestinal epithelial cells (IEC)^[108]. This uncharacterized factor released into the serum of 7 d resected rats significantly increased EGF-R protein expression. Additionally, the proliferative response in IEC's was abolished following the addition of a specific EGFR inhibitor. It would be intriguing to explore if there is cross talk between GLP-2, which is significantly increased following intestinal resection, and the EGFR signaling pathway. The basis for this speculation was

discussed earlier and is depicted in Figure 3B.

PACAP and VIP

All but one of the members of the PACAP/glucagon superfamily, GIP, have been found as protein and/or mRNA in the brain and are thus classified as neuropeptides^[34]. For an excellent review of this family of peptides, see Sherwood *et al*^[34]. All of these peptides are found in the gut and signal through 7-TM G-protein coupled receptors. In humans, the present members include PACAP, glucagon, glucagon-like peptide-1, growth hormone releasing hormone, vasoactive intestinal peptide (VIP), secretin, glucose-dependent insulinotropic peptide (GIP), and GLP-2^[34]. Both VIP and PACAP have a widespread distribution and are known to affect neural, circulatory, gastrointestinal, endocrine, and immune systems. There is evidence that VIP and PACAP administration reduces apoptosis and promotes the survival of neural cells by inducing *bcl-2* activity. Increased *bcl-2* activity is associated with an inhibition of caspase-3 activity and decreased cytoplasmic cytochrome C release^[34]. Moreover, the BH4 domain of the pro-survival protein *bcl-2* both binds to the C-terminus of Apaf-1 on caspase-9, thus inhibiting caspase-induced apoptosis, as well as directly or indirectly preventing the release of cytochrome C from the mitochondria^[109,110]. Similar results were attained following the activation of GLP-2R signaling by GLP-2 administration in transfected cells treated with cyclohexamide or irinotecan. These data show that there was an inhibition of apoptosis, reduced caspase activation, and decreased mitochondrial cytochrome C release^[57,84]. An additional parallel of these peptides is revealed following recent studies suggesting that GLP-2 is a vasodilator^[111,112]. PACAP and VIP are both established as potent vasodilators^[34].

At physiological concentrations, VIP and GLP-2 are both ineffective in stimulating cell growth directly in cell culture. Perhaps the intact ENS might need to be activated first as VIP receptors, and likely GLP-2 receptors, are located on enteric neurons^[34,51]. It would be interesting to examine the growth-promoting potential of the ENS on epithelial cells *in vivo*.

There are conflicting results regarding plasma and tissue levels of VIP following small bowel resection and in studies examining SBS. These differences may be species-specific as following intestinal resection, dogs and pigs have a significant reduction in VIP levels, whereas in humans, there is either an elevation or no change in plasma VIP levels^[113-116]. There is no evidence that VIP and PACAP administration is directly trophic for the small bowel thus a role for VIP in small bowel resection in the rat has not been described.

Peptide YY and Cholecystokinin

Further study is required when looking at the relationship of other co-localized mediators that are released from the L-cell. Peptide tyrosine tyrosine (PYY) is a 36 amino acid gastrointestinal peptide present in endocrine L-cells of the ileal, colonic, and rectal mucosa^[117-121]. PYY is co-localized to some degree with GLP-1/GLP-2 in the L-cells^[122,123],

moreover, it generates many of the physiological actions that are also attributed to GLP-2. There are data that both GLP-2 and PYY: slow gastric emptying and intestinal transit; production rates in rats and humans are increased following intestinal resection, production rates are influenced by luminal nutrients in the hindgut; release might be influenced by a proximal gut hormone or perhaps a neuroendocrine mechanism^[51,124-133]. In support of the latter, our results examining postprandial GLP-2 release^[15], together with reports of the meal-stimulated PYY response^[133], demonstrate that there are significant increases in the serum levels of both peptides in less than 15 min. Intestinal transit times are not that rapid, thus these early increases probably are not attributable to luminal nutrients in the distal intestine. Neuroendocrine factor induction of the early release of PYY was described in a study by Greeley *et al*^[121]. They administered a meal to dogs and then diverted the entire meal out of the proximal intestine via the creation of a stoma. Thus, there was no possibility of nutrient stimulation of PYY containing L-cells in the distal gut. Interestingly, there was still significant increase in plasma PYY levels. In these same dogs, the removal of the ileum, colon, and rectum, resulted in no increase in plasma PYY levels in response to a meal. These findings replicate a human study previously discussed in this paper, but instead of PYY, GLP-2 levels in patients that received a colectomy were significantly reduced^[47]. This is certainly supportive of either a blood-borne hormone or a neural pathway in the generation of the early release of PYY and most assuredly, GLP-2. Furthermore, we have observed that rats with 20 cm of jejunum diverted out an abdominal stoma (i.e. no possibility of meal contents directly contacting the distal bowel) still had a significant increase in post-prandial GLP-2 levels-unpublished data-(Figure 5).

Several proximal gut hormones that are rapidly secreted following the ingestion of a meal have been tested (i.e., gastrin, glucose dependent insulinotropic peptide, secretin, neurotensin) and found to be ineffective in stimulating an increase in PYY^[121]. Currently, no published literature has shown that these can stimulate GLP-2 release. One potential agonist, cholecystokinin (CCK), induces a dose-dependent release of PYY, and this CCK-induced PYY release, can be blocked by a CCK antagonist^[121]. As CCK induces PYY production from L-cells, and having established that PYY and GLP-2 are co-localized, potentially, CCK might also be a foregut hormone responsible for stimulating the early release of GLP-2. It would be worthwhile to examine whether CCK also triggers GLP-2 secretion.

PYY administration has not shown that it is trophic to the gastrointestinal tract directly and it is unlikely that it is involved in the adaptive response following intestinal resection, though a reduction in intestinal transit may secondarily generate increased intestinal growth. These areas have not been explored. Both GLP-2 and PYY are inactivated by dipeptyl peptidases^[73,76], thus additional studies of SBS should explore the relationship between serum and intestinal tissue levels of GLP-2, PYY, and DPP-IV.

Polyamines

Polyamines are present in prokaryotes, plants and animals. The polyamines putrescine and its derivatives spermidine and spermine are found in nearly all cells of higher eukaryotes^[134,135]. The major role of polyamines in most cell types is to stimulate cell proliferation and thus is considered essential for life^[135,136]. The inhibition of polyamine biosynthesis blocks cell growth^[136,137]. The increase in intracellular polyamine levels and in the activity of ornithine decarboxylase (ODC), one of the rate-limiting enzymes in the strictly controlled polyamine biosynthetic pathway^[138], are associated with rapid growth rates^[139,140]. ODC activity in the rat small intestinal mucosa is increased following small bowel resection^[134,138], parasitic or enteropathogenic bacteria-induced small intestinal inflammation or colitis^[141,142], ischemia reperfusion^[143], and following partial obstruction of the lumen. All of these instances are associated with an increase in mucosal growth. Of clinical relevance, mucosal ODC activity in the colon and rectum has been reported to be significantly higher in both ulcerative colitis and Crohn's disease patients^[144]. In addition, evidence shows that polyamines are protective of DNA during the S-phase of the growth cycle^[135,145] and in part may explain the protective effects of GLP-2 following the administration of chemotherapeutic agents.

There is a rise in plasma enteroglucagon levels that precedes the activation of ODC^[143]. Thus it could be speculated that an increase in GLP-2 might initiate the activation of ODC leading to the repair of injured intestinal mucosa. If so, it would not be unreasonable for the growth promoting and protective role of GLP-2 in the small bowel to act through an ODC-dependent formation of polyamines. Considering that the trophic effects of many growth-promoting gut hormones are blocked following ODC or polyamine synthesis inhibition^[134,138], it would not be surprising if this pathway is involved as a downstream effector of GLP-2 induced intestinal growth.

Intestinal carcinogenesis

A great deal of attention has focused on the potential for cancerous growth attributable as a side effect following the application of growth factors. Recent results cautioning restraint regarding the utility of GLP-2 was indicated as GLP-2, and to a greater degree, a GLP-2 analog, promoted an increase in the growth of mucosal neoplasms in mice pre-induced to form colonic tumours^[146]. Furthermore, there was a significant increase in tumour load in the mice treated with either GLP-2, or the synthetic Gly2-GLP-2 analogue. There is also evidence that GLP-2R mRNA transcripts can be detected in two human cervical carcinoma cell lines^[56]. But does the application of growth factors such as GLP-2 or EGF always trigger neoplastic growth? Could the application of growth factors prevent the development of intestinal neoplasms? Perhaps the primary factor involved in neoplastic growth is not necessarily the addition of the growth factor, but rather a dysfunction in downstream receptor (i.e., mutation) or signal transduction pathways. For example, there is evidence that mutations in cytoplasmic signaling elements involved in the EGFR signaling cascade result in an

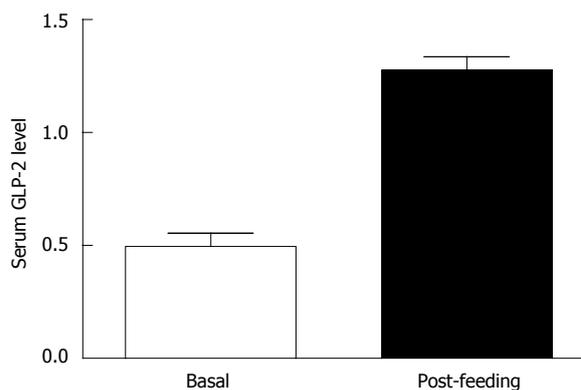


Figure 5 Neural or humoral mechanism involvement in the early release of GLP-2. The delivery of a meal significantly increases serum GLP-2 levels in rats with discontinuous small bowel (ANOVA, $P < 0.007$). The disruption of the continuity of the bowel (stoma creation) did not inhibit the initial nutrient-stimulated GLP-2 response. Thus, GLP-2 production is increased at least in part by neural and/or hormonal mechanisms. Serum GLP-2 levels reported as ng/mL.

alteration in signal transduction events that lead to changes in the downregulation/internalization of the EGFR^[147]. Perhaps what occurs in established oncogenic cells is that a mutation in an element of the signaling cascade prevents GLP-2- or EGF-induced receptor downregulation. This theory is supported by recent work that shows that an oncogenic form of c-Cbl/c-Cbl, which stabilizes and ubiquitinates the cytoplasmic region of the EGFR, is able to prevent EGF-induced EGFR down regulation thus bypassing degradative pathways^[148]. The consequences of this mutation are a bypass of receptor internalization and desensitization processes, an induction in the over expression of the EGFR, and finally, aberrant cell growth. A downstream defect in trafficking could potentially be the primary cause of neoplastic, abnormal cell growth, not necessarily the application of the growth factor.

In the case of the promotion of tumour size in mice following GLP-2 treatment as noted by Thulesen *et al.*, or potentially as in the CaCO-2, T84, HeLa cells^[56,58,59], what has occurred is that dysfunctional receptors and/or signal transduction pathways are already present within/on these cell types. As with any neoplastic condition, the application of growth factors is always a risk. Potentially the application of growth factors prior to the development of gastrointestinal neoplasms, such as during the early stages of Crohn's disease or ulcerative colitis, would be beneficial in halting the progress of prolonged inflammation. It is well established that prolonged intestinal inflammation often secondarily leads to the development of mutations, cell dysfunction, and carcinogenic growth.

CONCLUSIONS

The trophic effect of GLP-2 on the intestine demonstrates that it is possible to modify or accelerate the process of intestinal adaptation, which would potentially make GLP-2 useful for the treatment of short bowel syndrome. Evidence shows that increases in GLP-2 production following intestinal resection are significantly correlated with intestinal adaptation. The association between an increase in nutrient malabsorption and GLP-2 production

following intestinal resection has led to speculations that GLP-2 may act to control the nutrient absorptive capacity within the bowel. The increased availability of circulatory GLP-2 might then stimulate proximal mucosal growth resulting in enhanced nutrient absorptive capacity in the remaining intestinal remnant. Moreover, GLP-2 administration maintains epithelial barrier function and increases both crypt cell proliferation and weight gain in the absence of enteral nutrients. This supports the further development of GLP-2 as a therapeutic strategy that could enhance intestinal adaptation and reduce the consequences of parenteral feeding.

Although significant progress has been made towards elucidating the mechanisms mediating the trophic effects of GLP-2, several formidable challenges lie ahead. The biological and signaling role of the GLP-2R has not been fully defined, thus the mechanistic pathway (s) by which GLP-2 induces adaptive change in the small bowel presently remain elusive. Data showing that GLP-2 administration significantly increases cell proliferation in the absence of the characterized GLP-2R strongly suggest that GLP-2's trophic effect might also include activation of either an uncharacterized GLP-2R or the transactivation of other receptor/signaling paradigms. Another key goal should be in determining the mechanism involved in the early release of GLP-2 following a meal or in malabsorptive states. Lastly, an emergent area of interest is the potential of GLP-2 to induce stimulation of the ENS as an intermediate in the regulation of intestinal growth. Regardless, there is a very significant and biologically important effect of GLP-2 on intestinal function and whole animal physiology that strongly supports further research into the use of GLP-2 as a therapy. The future use of GLP-2 therapy for the treatment of intestinal disorders is an enticing prospect; however, the promise of these initial results must be tempered as we await confirmatory data from clinical trials.

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GASTRIC CANCER

Alphastatin downregulates vascular endothelial cells sphingosine kinase activity and suppresses tumor growth in nude mice bearing human gastric cancer xenografts

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Abstract

AIM: To investigate whether alphastatin could inhibit human gastric cancer growth and furthermore whether sphingosine kinase (SPK) activity is involved in this process.

METHODS: Using migration assay, MTT assay and Matrigel assay, the effect of alphastatin on vascular endothelial cells (ECs) was evaluated *in vitro*. SPK and endothelial differentiation gene (EDG)-1, -3, -5 mRNAs were detected by reverse transcription-polymerase chain reaction (RT-PCR). SPK activity assay was used to evaluate the effect of alphastatin on ECs. Matrigel plug assay in nude mice was used to investigate the effect of alphastatin on angiogenesis *in vivo*. Female nude mice were subcutaneously implanted with human gastric cancer cells (BGC823) for the tumor xenografts studies. Micro vessel density was analyzed in Factor VIII-stained tumor sections by the immunohistochemical SP method.

RESULTS: *In vitro*, alphastatin inhibited the migration and tube formation of ECs, but had no effect on proliferation of ECs. RT-PCR analysis demonstrated that ECs expressed SPK and EDG-1, -3, -5 mRNAs. *In vivo*, alphastatin sufficiently suppressed neovascularization of the tumor in the nude mice. Daily administration of alphastatin produced significant tumor growth suppression. Immunohistochemical studies of tumor tissues revealed decreased micro vessel density in alphastatin-treated animals as compared with controls.

CONCLUSION: Downregulating ECs SPK activity may be one of the mechanisms that alphastatin inhibits gastric cancer angiogenesis. Alphastatin might be a useful and relatively nontoxic adjuvant therapy in the treatment of gastric cancer.

INTRODUCTION

Angiogenesis, the development of new blood vessels from pre-existing endothelium is a critical process in many physiological and pathological conditions including embryonic development, organ regeneration, chronic inflammation and solid tumor growth. The process of formation of new blood vessels is complex and involves several discrete steps, such as ECs spreading, migration, proliferation and morphological differentiation of endothelial cells to form tubes. In the last three decades, considerable research has demonstrated that tumor growth and metastasis requires angiogenesis, and micro vascular endothelial cells recruited by the tumor have become an important second target in cancer therapy^[1-3]. This is the major reason why angiogenesis has attracted recent attention in the field of pharmacological research. The key for the development of such an angiostatic therapy is to develop useful angiogenesis inhibitors. A number of research groups have shown that various substances are effective in the inhibition of angiogenesis and/or in the treatment of angiogenic diseases like cancer at the experimental animal model level. Various angiostatic factors such as endostatin, angiostatin and thrombospondin have been identified and can block various steps in the tumor angiogenesis pathway. The application of these antiangiogenic agents for cancer treatment is being evaluated through clinical trials and many new angiogenesis inhibitors were reported^[4-6]. In previous studies, it was reported that alphastatin (24 amino acids) derived from the amino terminus of human fibrinogen could inhibit the growth of murine colonic adenocarcinoma. Significant reduction in tumor volume was found from the fifth day of administration and maintained for 12 d of intraperitoneal injection^[7]. S1P, formed through activation of SPK activity,

is a bioactive sphingolipid metabolite abundantly stored in platelets. Recently, S1P has been targeted for its potential roles in angiogenesis. It may stimulate DNA synthesis and chemotactic motility of ECs, and also induce tube formation of ECs on Matrigel. Further investigation indicated that S1P predominantly induces angiogenesis via endothelial differentiation gene (EDG), a family of Gi protein-coupled receptor. Activation of EDG receptors triggers several signaling pathways by pertussis toxin (PTX)-sensitive Gi protein. Moreover the signaling pathways activated by S1P have been extensively studied in various cell types. All of these proved that S1P may be a novel mechanism during angiogenesis.

In the present study, we attempt to extend the previous study to observe whether alphastatin inhibits human gastric cancer growth. Importantly, we first report that alphastatin suppress angiogenesis through downregulating ECs SPK activity and reducing ECs S1P level.

MATERIALS AND METHODS

Materials

Human umbilical vein ECs (HUVECs) and human gastric cancer cells (BGC823) were obtained commercially from ATCC. High glucose Dulbecco's modified Eagle's medium (DMEM) and bovine serum albumin (BSA, 1 g/L) were obtained from Sigma chemicals (St. Louis Mo, USA). Growth factor reduced (GFR) Matrigel was purchased from Becton Dickinson (San Jose, Calif, USA). Alphastatin was synthesized by New England Biolabs (Beverly, MA) using standard peptide synthesis techniques and purified to more than 95% using high performance liquid chromatography (HPLC). Hepatocyte growth factor (HGF) was purchased from Peprotech EC Ltd. Transwell plate was purchased from Corning Costar (Cambridge, MA).

Endothelial cells migration assay

HUVECs and BGC823 cells were cultured in DMEM containing 100 mL/L FBS and 10 g/L penicillin-streptomycin in a 50 mL/L CO₂ incubator at 37°C. Assessment of ECs migration was performed as recently described with minor modifications^[8]. HUVECs were dispersed into homogeneous single cell suspensions after trypsinization. These cells were extensively washed with DMEM containing 1 g/L acid-free BSA and resuspended in the same medium. HUVECs (10⁵) were dispersed onto the upper chamber of Transwell compartment with 8 µm pore size filter. The cells were allowed to adhere for 1 h at 37°C. The medium in the lower chamber was removed and replaced by migration medium containing HGF, HGF plus alphastatin or medium alone. Migration was allowed to proceed for 4 h at 37°C. The remaining cells attached to the upper surface of the filters were carefully removed with cotton swabs. Migrated cells were stained with crystal violet and finally examined by light microscopy. The numbers of migrated cells in at least 10 consecutive fields were evaluated and the average was calculated. Data were expressed as the mean ± SD of the number of migrated cells per field in 5 separate experiments.

Endothelial cells proliferation

HUVECs proliferation was assayed as described previously^[9] using standard MTT assay. Alphastatin was dissolved in PBS and diluted in DMEM medium. Cells were incubated in their complete medium containing 100 mL/L FBS, the complete medium plus alphastatin or the starvation medium without FBS. The cells were seeded into 96-well micro titer plates at 5 × 10⁷ cells/L in the presence of various concentrations of alphastatin for 24, 48, 72, and 96 h. At each time point, a quarter volume of MTT solution (2 g MTT/L phosphate-buffered saline) was added to each well and each plate was incubated for 4 h at 37°C resulting in an insoluble purple formazan product formation. The medium was aspirated and the precipitates dissolved in 100 µL of dimethyl sulfoxide (DMSO) buffered at PH 10.5. Absorbance at 490 nm was determined using R450 microplate reader (Bio-Rad, USA). Each sample was assayed in five duplicates and repeated at least three times. In order to validate the cytotoxicity of alphastatin, the cells were seeded at a density of 5 × 10⁴ cells per well in full growth medium in the absence or presence of alphastatin. After 24 h, the data was collected using CellTiter 96[®] AQueous One Solution Reagent Kit (Promega, USA).

Endothelial cells tube formation on Matrigel

Endothelial tube formation on Matrigel was conducted as described previously^[10]. Matrigel (10 g/L per well) at 4°C was added to a twenty-four-well plates and then allowed to polymerize at 37°C for 1 h. HUVECs (5 × 10⁴ cells) were seeded on Matrigel in 1 ml of growth medium and various concentrations of alphastatin. The plates were incubated at 37°C for 18 h. All conditions were triply performed. The formation of tube-like structures by HUVECs was analyzed by fluorescence microscope at × 100 magnification and the total area of tubular structures in five random microscopic fields per well was analyzed using Image tools package 3.0.

Reverse transcription polymerase chain reaction (RT-PCR)

After treated with various dose of alphastatin, total RNA was obtained from HUVECs using the Trizol Reagent kit (Invitrogen, USA). Two µg of total RNA was converted to cDNA by treatment with 100 units of reverse transcriptase and 0.5 µg of oligo-dT primer in 50 mmol/L Tris-HCl (pH 8.3), 75 mmol/L KCl, 3 mmol/L MgCl₂, 0.1 mol/L DTT and 1 mmol/L dNTP at 42°C for 1 h. The reaction was terminated by heating at 70°C for 15 min. Two µL of the cDNA mix was used for enzymatic amplification. Polymerase chain reaction was performed in 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.3), 1.5 mmol/L MgCl₂, 0.2 mmol/L dNTP, 2.5 units of Ex *Taq* DNA polymerase and 0.1 µmol/L for each of primers such as SPK, EDG-1, EDG-3 and EDG-5. The reaction mixture was repeated for 35 cycles, as heated at 95°C for 45 s, annealed at 57°C for 30 s and extended at 72°C for 60 s. The primers used were 5'ATGCACGAGG TGGTGAACG3' (sense) and 5'GGAGGCAGGTGTCTTGG AAC3' (antisense) for the SPK (426 bp); 5'CCGCAAGAACATTTCCAAG (sense) and 5'ACCCACCAACACCCGACAC (antisense) for EDG-1 (608 bp); 5'CCTGC GGGAGCATTA CCA

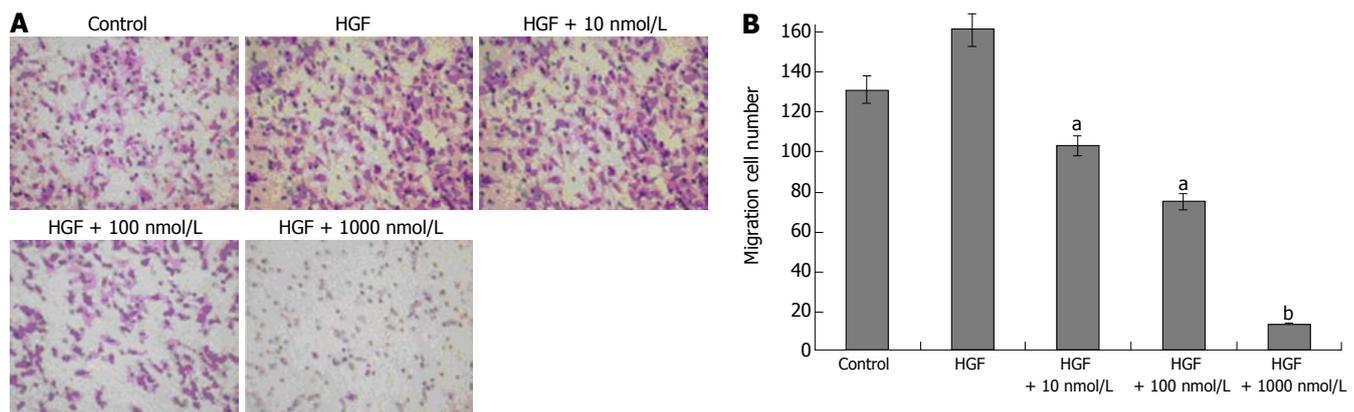


Figure 1 Alphastatin inhibit HUVECs migration induced by HGF (crystal violet stain, $\times 100$). All data shown as mean \pm SD. ^a $P < 0.05$; ^b $P < 0.01$ vs control group.

(sense) and 5'CACCTTACGGCTGCTGGAC (antisense) for EDG-3 (637 bp); 5'AAGTTCCACTCGGCAATGTAC (sense) and 5'GCAGC CAGCAGACGA TAAA (antisense) for EDG-5 (556 bp).

Measurement of sphingosine kinase activity

After various treatments, HUVECs were washed twice with PBS and harvested by scraping in 0.1 mol/L Tris-HCl buffer (pH 7.4) containing 200 mL/L (v/v) glycerol, 1 mmol/L mercaptoethanol, 1 mmol/L EDTA, 1mmol/L Na_3VO_4 , 15 mmol/L NaF, 10 mg/L leupeptin and aprotinin, 1 mmol/L phenylmethylsulfonyl fluoride and 0.5 mmol/L 4-deoxy pyridoxine. Cells were lysed by freezing and thawing. Cytosolic fractions were prepared by centrifugation at 12000 g for 30 min. One hundred and eighty milliliters of cytosol was incubated with 10 μL of sphingosine (1 mmol/L, dissolved in 50 g/L Triton X-100), 10 μL [γ -³²P] ATP (20 mmol/L) containing MgCl_2 (200 mmol/L) for 15 min. Sphingosine kinase activity was measured as previously described^[11-13].

In vivo Matrigel plug assay

Matrigel plug assay was performed as previously described^[14]. Briefly, nude mice were injected subcutaneously with 0.5 mL Matrigel. The injected Matrigel rapidly formed a single solid gel plug. After 7 d, the mice were euthanasia killed and Matrigel plug were removed. It was fixed with 50 g/L formaldehyde phosphate buffer saline and embedded in paraffin. The slides were routinely cut and stained with hematoxylin & eosin (HE). Capillaries were defined as tubular structures containing red blood cells.

Tumor cells implantation and pathology

The in-house and governmental animal protection committees approved all of the experiments and the animals were cared according to the guidelines for laboratory animals established by the Chinese government. Female athymic mice (Balb/c, nu/nu, 8 wk of age; weighing about 20 g) were maintained under clean room conditions in sterile rodent micro isolated cages. Animals received sterile rodent chow and water *ad libitum*. Human gastric cancer cells (BGC 823) were injected subcutaneously (SQ) into the right hind limb (2×10^6 cells in 100 μL of PBS). Tumor cell grew for 8 d until the tumor block established. Animals were ran-

domized for therapy when tumor volume reached 120-160 mm^3 . Tumor volume was determined three times weekly by direct measurement with calipers and was calculated by the formula "Volume (V) = length \times width² \times 0.52". Animals were intraperitoneally daily injected with alphastatin (0.25 mg or 2.5 mg/kg per day in 7 mice respectively) or PBS (control in 7 mice). The observations were terminated for ethical reasons when the tumor volume became large compared with the animal size. Tumor for histological analysis was harvested from three animals at d 12 after the start of antiangiogenic therapy. At the end of observation, tumor tissues were fixed in buffered formalin and were embedded in paraffin. Tissue slices (5 μm) were cut and stained with HE. To assess the tumor angiogenesis, immunohistochemical staining was performed using the anti-mouse Factor-VIII monoclonal antibody. Tumor angiogenesis was quantified by counting the number of positively stained microvessels in 10 randomly chosen fields at $\times 400$ magnifications.

Statistical analysis

The data were presented as mean \pm SD and analyzed by a statistical software of SPSS 10.0 for Windows program using Student's *t*-test and the analysis of variance (ANOVA). Significant differences were considered when $P < 0.05$.

RESULTS

Vascular endothelial cell migration and proliferation

Vascular endothelial cell migration is critical for tumor angiogenesis. To determine the effects of alphastatin on migration of HUVECs induced by chemoattractant media (HGF), we counted the number of cells that had migrated to the bottom of the Transwell membrane. Alphastatin significantly inhibited HUVECs migration in response to HGF in a dose-dependent manner (103 ± 4 and 75 ± 3 vs 131 ± 4 , $P < 0.05$; 13 ± 1 vs 131 ± 4 , $P < 0.01$, Figure 1). Alphastatin had no effect on cell proliferation even up to 2000 nmol/L for 96 h (Figure 2). The cytotoxicity of alphastatin was assessed and no detectable cytotoxic effect at those doses was found *in vitro* (data not shown).

Tube formation by HUVECs

When HUVECs were placed on growth factor-reduced

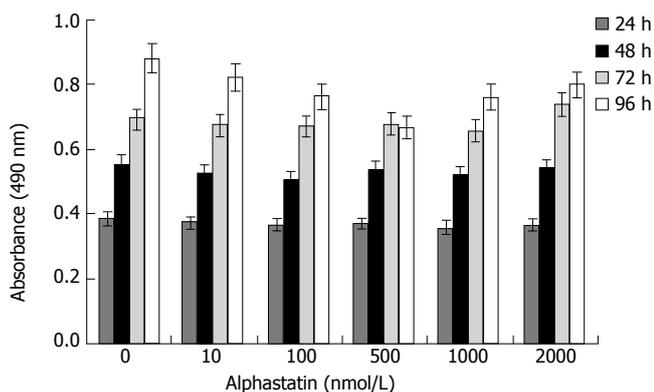


Figure 2 Alphastatin has no effect on HUVECs proliferation.

Matrigel surface, they formed a branching and anastomosing network of capillary like tubules with multicentric junctions over 18 h. Alphastatin inhibited the formation of tubular networks induced by PMA (25 µg/L) on Matrigel in a dose-dependent manner (Figure 3). A remarkable inhibition of tube formation was observed in the presence of 100 nmol/L (1508.96 ± 29.89 µm² *vs* 2150 ± 31.05 µm², *P* < 0.05). At the same size field, there was longer distance between tubes and less tubules at alphastatin-treated group. Tube formation was quantitatively estimated by measuring the area covered by the tube network using the image analysis program.

SPK mRNA, EDG-1, EDG-3, and EDG-5 expression

Before measurement of SPK activity, RT-PCR was carried out using specific primers for SPK, EDG-1, EDG-3 and EDG-5 to assure expression of them in HUVECs. It was revealed that SPK, EDG-1, EDG-3 and EDG-5 were expressed in HUVECs (Figure 4). Whether alphastatin could change HUVECs SPK activity is a mystery. HUVECs were starved overnight in serum-free medium before the addition of alphastatin. Then, these cells were stimulated with various concentration of alphastatin for different times. Alphastatin could downregulate SPK in a dose-dependent manner. When HUVECs were treated with alphastatin at a concentration of 100 nmol/L, the cellular SPK activity reached a minimum value at 6 h (Figure 5). These data showed that alphastatin leads to downregulation of SPK through stimulation of HUVECs.

New capillaries formation

Matrigel was injected into nude mice with or without alphastatin. Reduction in the number of blood vessels was observed at 100 nmol/L alphastatin group (Figure 6). Sections of experimental tumors were stained with antimouse Factor-VIII antibody.

Microvessel density was less in alphastatin-treated group than in PBS group (Figure 7).

Gastric cancer growth in nude mice

For tumor growth quantification, we used the human BGC823 gastric cancer cell lines. After injecting tumor cells, tumors were allowed to grow until the block established (10 d). Then mice were randomized and divided into control group (PBS: *n* = 7) and therapy group

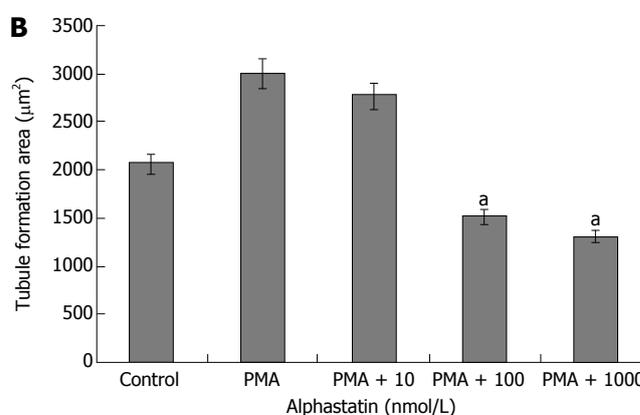
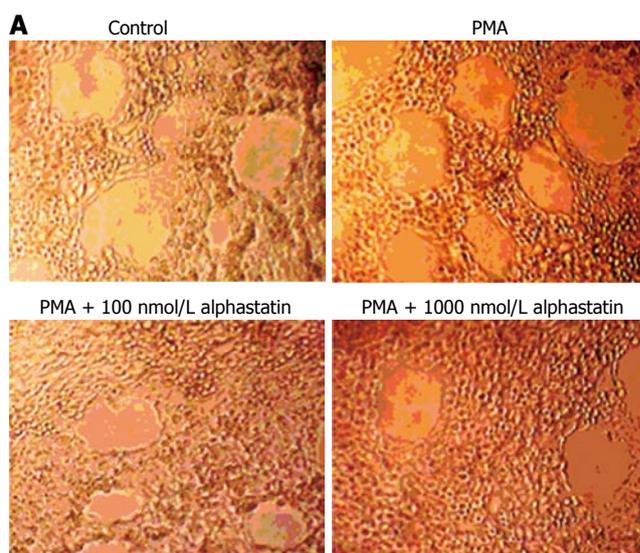


Figure 3 Alphastatin inhibit HUVECs tubule formation in response to PMA. All data shown as mean ± SD. ^a*P* < 0.05 vs control group.

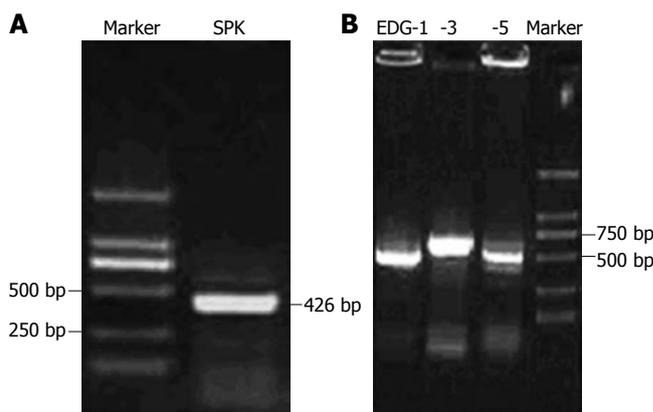


Figure 4 Expression of SPK, EDG-1, EDG-3, EDG-5 in HUVECs.

(alphastatin: *n* = 7). The tumor in control group steadily grew up to a final tumor volume of 1771 ± 262 mm³ over the same period as the therapy group. However, the growth rate of the tumor in the therapy group was significantly reduced between d 18 and d 26 (*P* < 0.05 and *P* < 0.01, Figure 8), and a final tumor volume was only 1145 ± 114 mm³ and 612 ± 173 mm³ respectively. Moreover, alphastatin injection appeared to be well tolerated *in vivo*

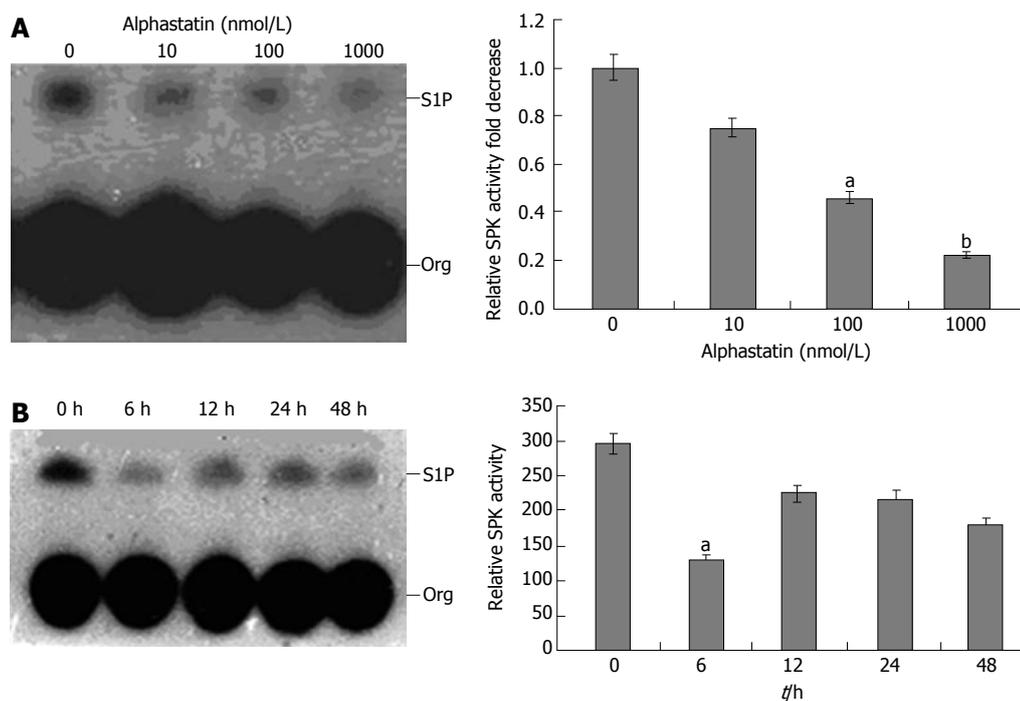


Figure 5 Alphastatin downregulate HUVECs SPK activity in a dose-dependent manner. HUVECs SPK activity reached a minimum value at 6 h. All data shown as mean \pm SD. **A:** ^a $P < 0.05$; ^b $P < 0.01$ vs 0 nmol/L alphastatin; **B:** ^a $P < 0.05$ vs 0 h group.

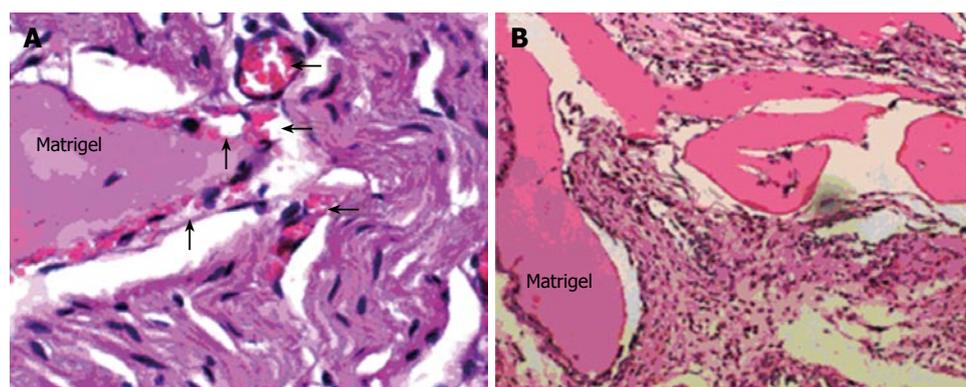


Figure 6 Alphastatin inhibit new capillaries formation on Matrigel plug (original magnification $\times 100$). **A:** Many newly formed capillaries full of red blood cells (arrow); **B:** Granuloma formation without significant neovasculation.

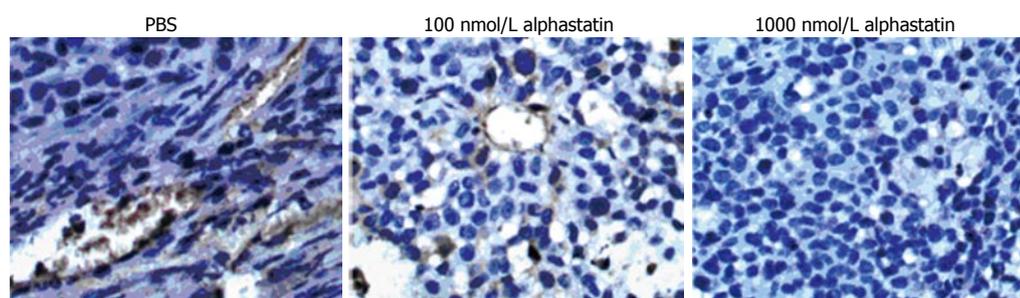


Figure 7 Factor-VIII staining revealed decreased blood vessel density in alphastatin-treated group vs PBS group ($\times 400$).

and had no significant effect on animal body mass and general condition.

DISCUSSION

Previous studies show that alphastatin is a potent antiangiogenic agent^[7]. However, little is known about whether it has any effect on human derived tumors and what mechanism related to the antiangiogenic activity of alphastatin. In the present study, we therefore tried to solve the question above. Gastric cancer is one of the

most common malignancies in Asia. However, so far there is no effective therapeutic measure for this highly malignant disease. Recent studies have demonstrated that angiogenesis is a prerequisite for development and growth of different tumors^[15-18]. Anti-angiogenic targeting of the neovasculation within tumors is considered one of the most promising strategies in the search for novel antineoplastic therapies^[19]. Strategies for anti-angiogenic drugs were described as follows: (1) interference with endothelial cell migration and proliferation; (2) interference with endothelial cell tubule formation and (3)

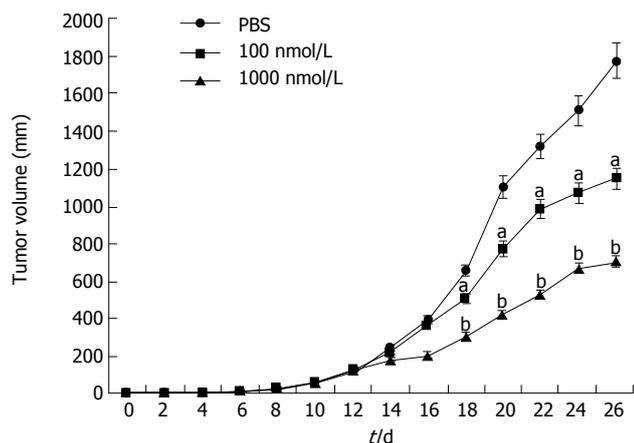


Figure 8 Animals injected with alphastatin exhibited a significantly reduced tumor volume. All data shown as mean \pm SD. ^a $P < 0.05$; ^b $P < 0.01$ vs PBS group.

interference with some factors involved in angiogenesis pathway^[20-25]. The present study shows that alphastatin inhibits angiogenesis *in vitro* and *in vivo*. Furthermore, for the first time we described that alphastatin could suppress human gastric cancer growth in nude mice through downregulating vascular endothelial cell SPK activity. In migration assay, we chose HGF as chemoattractant media because it is a multifunctional factor regulating cell growth, motility and migration for a variety of cell types including endothelial cell, epithelial cell and hepatocyte. Its effect of chemotaxis is greater than vascular endothelial growth factor (VEGF). Duan *et al* showed that HGF/c-Met activates SPK *via* ERK1/2 and PI3K pathways. SPK activation plays an important regulatory role in HGF-induced migration of endothelial cells^[26]. Lee *et al* showed that S1P strongly induces the formation of tube network of HUVECs which has the characteristics of strong intercellular interaction and sustained survival of large population of cells on Matrigel^[11]. These morphological changes of HUVECs may occur mainly through binding of S1P with EDG-1. In addition, S1P in part and indirectly stimulates HUVECs proliferation through direct secretion of angiogenic factors, because an increase in the expression of VEGF mRNA was detected within 24 h after treatment of HUVECs with S1P^[27-29]. In the present study, alphastatin inhibited the migration and tube formation of HUVECs, but no significant effect on cell proliferation even up to 2000 nmol/L. It was in SPK activity assay shown that alphastatin down regulated HUVECs SPK activity and reduced cellular S1P production. From the data above, it could be concluded that alphastatin inhibited the process of angiogenesis through interference with HUVECs SPK activity.

In the *in vivo* Matrigel plug assay, there was only granuloma formation without significant neovasculature in alphastatin group. Therefore, the antiangiogenic action of alphastatin *in vivo* may be due to its inhibitory effects on endothelial cells stimulated by VEGF as shown in the present study^[5]. In our tumor model, the BGC823 tumors continued to grow over the 12-d injection period, whereas nude mice injected daily with alphastatin demonstrated significant reduction in tumor volume from

d 8 of administration and this was maintained for 14 d of injection. The inhibition was in a dose-dependent manner. Immunohistochemical studies of tumor tissues revealed decreased microvessel density in alphastatin-treated animals as compared with control group. It is also likely that alphastatin down regulated SPK activity and reduced endothelial cell S1P production, resulted in inhibition of angiogenesis.

In conclusion, our current studies provide evidence for anti-angiogenic activities of alphastatin. It induced human gastric cancer vascular growth ceasing *in vivo*, inhibited tube formation on Matrigel and disrupted HUVECs migration. More importantly, the inhibition of angiogenesis was through down regulating HUVECs SPK activity and this may be a novel mechanism that alphastatin exerts antiangiogenic activity. All of these findings hint that, as a new anti-angiogenic agent, alphastatin might be a prototype anti-tumor drug.

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Effects of inositol hexaphosphate on proliferation of HT-29 human colon carcinoma cell line

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Abstract

AIM: To investigate the effects of inositol hexaphosphate (IP₆) on proliferation of HT-29 human colon carcinoma cell line.

METHODS: Cells were exposed to various concentrations (0, 1.8, 3.3, 5.0, 8.0, 13.0 mmol/L) of IP₆ for a certain period of time. Its effect on growth of HT-29 cells was measured by MTT assay. The expressions of cell cycle regulators treated with IP₆ for 2 d were detected by immunocytochemistry.

RESULTS: IP₆ inhibited the HT-29 cell growth in a dose- and time-dependent manner. Analysis of cell cycle regulator expression revealed that IP₆ reduced the abnormal expression of P53 and PCNA and induced the expression of P21.

CONCLUSION: IP₆ has potent inhibitory effect on proliferation of HT-29 cells by modulating the expression of special cell cycle regulators.

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Key words: Phytic Acid; Colonic neoplasms; Cell proliferation

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INTRODUCTION

Colorectal cancer is the second most frequent cancer in Western countries^[1], and the third leading cause of cancer deaths in the United States^[2]. In China, the mortality

rate of colorectal cancer is the fourth to sixth leading cause of cancer deaths^[3]. Epidemiological studies have shown that high fiber foods, such as fruits, vegetables, whole grains and cereals, may protect against colorectal cancer^[4,8]. Animal studies have shown that wheat bran has protective effect against colorectal cancer^[9-14], which is attributed mostly to its high fiber content. Interestingly, many of the proposed protective mechanisms of wheat bran fiber, such as decreased transit time^[15], increased bulk^[16] and fermentation^[17], are analogous to those of inositol hexaphosphate (IP₆ or phytic acid), which is a major fiber-associated component of wheat bran^[18]. In some epidemiological studies, colorectal cancer-protective effect of fiber foods rich in IP₆, such as wheat bran has been observed^[18], indicating that IP₆ may protect against colorectal cancer.

IP₆ is a naturally occurring polyphosphorylated carbohydrate, found in plants, particularly in cereals and legumes (0.4%-6.4%)^[19]. It consists of a myo-inositol ring with six dihydrogen phosphate groups, assuming a chair conformation in dilute solution^[20]. This unique structure empowers IP₆ with a chelating capacity of binding to polyvalent (both mono and divalent) cations. Some of these metal ions such as magnesium and zinc play an important role in stimulation of cellular proliferation^[21]. This molecule is related to human health as an anti-nutrient. However, during the last decades it has been shown that IP₆ is also widely distributed in animal cells and tissues at substantial levels^[22,23]. Especially, a strong anti-cancer activity of IP₆ has been demonstrated both *in vivo* and *in vitro*^[24]. IP₆ exerts its anti-cancer activity by entering into cellular inositol hexaphosphate pool and affecting common cellular signal transduction pathways^[24,25], but its mechanisms of action are still not completely understood.

This study was to examine the effect of IP₆ on growth of HT-29 human colon carcinoma cell line. The expressions of cell cycle regulators were assessed after IP₆ treatment.

MATERIALS AND METHODS

Chemicals

IP₆ (a dodecasodium salt from rice) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) were purchased from Sigma (St Louis, MO, USA). DMEM/Ham F12 culture medium, fetal bovine serum and trypsin were from Gibco BRL (Grand Island, NY, USA). Rabbit polyclonal antibody to human P53, mouse monoclonal antibodies to human P21, PCNA and SP

histostain-plus kits were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell lines, culture conditions and IP₆ treatment

The HT-29 human colon carcinoma cell line was obtained from Xiehe Medical University (Beijing, China). Cells were grown in DMEM/Ham F12 medium supplemented with 10% fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 g/L) under standard culture conditions (37°C, 950 mL/L humidified air and 50 mL/L CO₂). A stock solution of 100 mmol/L IP₆ in distilled water was freshly prepared each time before use, the pH was neutralized with NaOH and sterilized by passing through a 0.22 μmol/L membrane filter. Dilutions of 1.8, 3.3, 5.0, 8.0, 13.0 mmol/L in DMEM/Ham F12 medium were prepared from stock solution immediately before use. The DMEM/Ham F12 medium with equal volume of distilled water served as negative control.

MTT assay

Cell number was determined by colorimetric MTT assay^[24]. MTT was dissolved in PBS at 5 g/L, filter-sterilized, diluted in the DMEM/Ham F12 medium, giving a final concentration of 1.0 g/L. For growth assay, cells were plated in 96-well microtiter plates (Costar, Cambridge, MA, USA) at a density of 2×10^3 cells/well of 100 μL media. One of the IP₆ treatments was that cells were exposed to 1.8-13.0 mmol/L IP₆ for 6 h, 12 h, or 24 h, after which IP₆ was removed and cells were grown in media without IP₆. This treatment was given every other day and continued for 6 d. The other IP₆ treatment was that cells were treated with various concentrations of IP₆ continuously for 6 d, during which culture media were changed with fresh media every other day. At the indicated time the media were removed, 50 μL of MTT was added, and the incubation was continued for 4 h at 37°C. Individual cell viability was assessed by visualization of intracellular blue crystal formation by light microscopy. The precipitated formazan was dissolved with 150 μL of DMSO, and the absorbance was determined at 490 nm with a microplate autoreader (EL311sx, Bio-Tek Instruments, Inc., Winooski, VT). Cell growth assay was repeated three times.

Immunocytochemistry

Immunocytochemical staining for P53, P21 and PCNA was carried out by the standard streptavidin-peroxidase-biotin technique (SP technique) using SP kit. Cells were treated with 1.8-13.0 mmol/L IP₆ for 2 d and collected by a brief trypsinization and plated on slides. The cells were fixed in acetone at -20°C for 5 min. The endogenous peroxidase activity was quenched in a 3% solution of hydrogen peroxide for 15 min and blocked for 10 min. Cells were immunostained with monoclonal antibody (dilutions: P21 1:50, PCNA 1:80) and P53 polyclonal antibody (dilutions: 1:50) for 1 h at 37°C. After three further washes with PBS, a second biotinylated goat anti-rabbit or rabbit anti-mouse antibody was applied for 1 h at room temperature and then streptavidin conjugated to peroxidase was added. Following extensive washes with PBS, 3, 3'-diaminobenzidine was used for color development, and hematoxylin was used for

Table 1 P53 expression in IP₆-treated HT-29 cells (mean ± SD)

Concentration of IP ₆ (mmol/L)	Absorbance value
Control	0.6772 ± 0.0095
1.8	0.6161 ± 0.0203
3.3	0.5996 ± 0.0205
5.0	0.6067 ± 0.0130
8.0	0.5871 ± 0.0159
13.0	0.5817 ± 0.0158

counterstaining. The negative controls were performed by substituting the primary antibody with PBS. Hematoxylin-stained cells were examined under light microscope and photographed. Cells not counterstained were measured by VIDAS2.1 image analysis system for absorbance because hematoxylin staining could affect the image-analysis results. Three highly magnified visual fields which were not overlapped were randomly selected to measure the absorbance of each field. The mean absorbance was calculated.

Statistical analysis

The experimental results were repeated three times and expressed as mean ± SD. Statistical analysis was carried out using one-way ANOVA. $P < 0.05$ was considered statistically significant. Statistical analyses were performed using SPSS 11.5 (SPSS Inc, Chicago, IL, USA).

RESULTS

Effect of IP₆ on the growth of HT-29 cells

Continuous treatment with IP₆ inhibited the proliferation of HT-29 cells (Figure 1A). The absorbance value for each IP₆ group was lower than that of control group. At the same time point, the absorbance value decreased with increasing IP₆ concentration. The absorbance values for the 8.0 mmol/L and 13.0 mmol/L IP₆ groups decreased on d 6 ($P < 0.05$). The effects of discontinuous treatment with IP₆ on the growth of HT-29 cells are shown in Figures 1B-1D. The treatment with IP₆ for 24 h inhibited the cell growth. But neither 6 h nor 12 h treatment showed dose- or time-dependent inhibition effects though the absorbance value for each IP₆ group was lower than that for control group.

Effects of IP₆ on the expression of P53, P21 and PCNA

Compared to the control, the expression of P53 protein in HT-29 cells was decreased after 2 d of IP₆ treatment at different concentrations ($P < 0.05$) (Table 1 and Figure 2A, Figure 2B).

Compared to the control, treatment of HT-29 cells with IP₆ at various concentrations for 2 d increased the expression of P21 ($P < 0.05$) (Table 2 and Figure 2C, Figure 2D).

Compared to the control, the expression of PCNA decreased after treated with IP₆ at different concentrations for 2 d ($P < 0.05$) (Table 3 and Figure 2E, Figure 2F).

The absorbance values assayed by MTT after IP₆ treatment for different periods of time are listed in Tables 4, 5, 6, and 7.

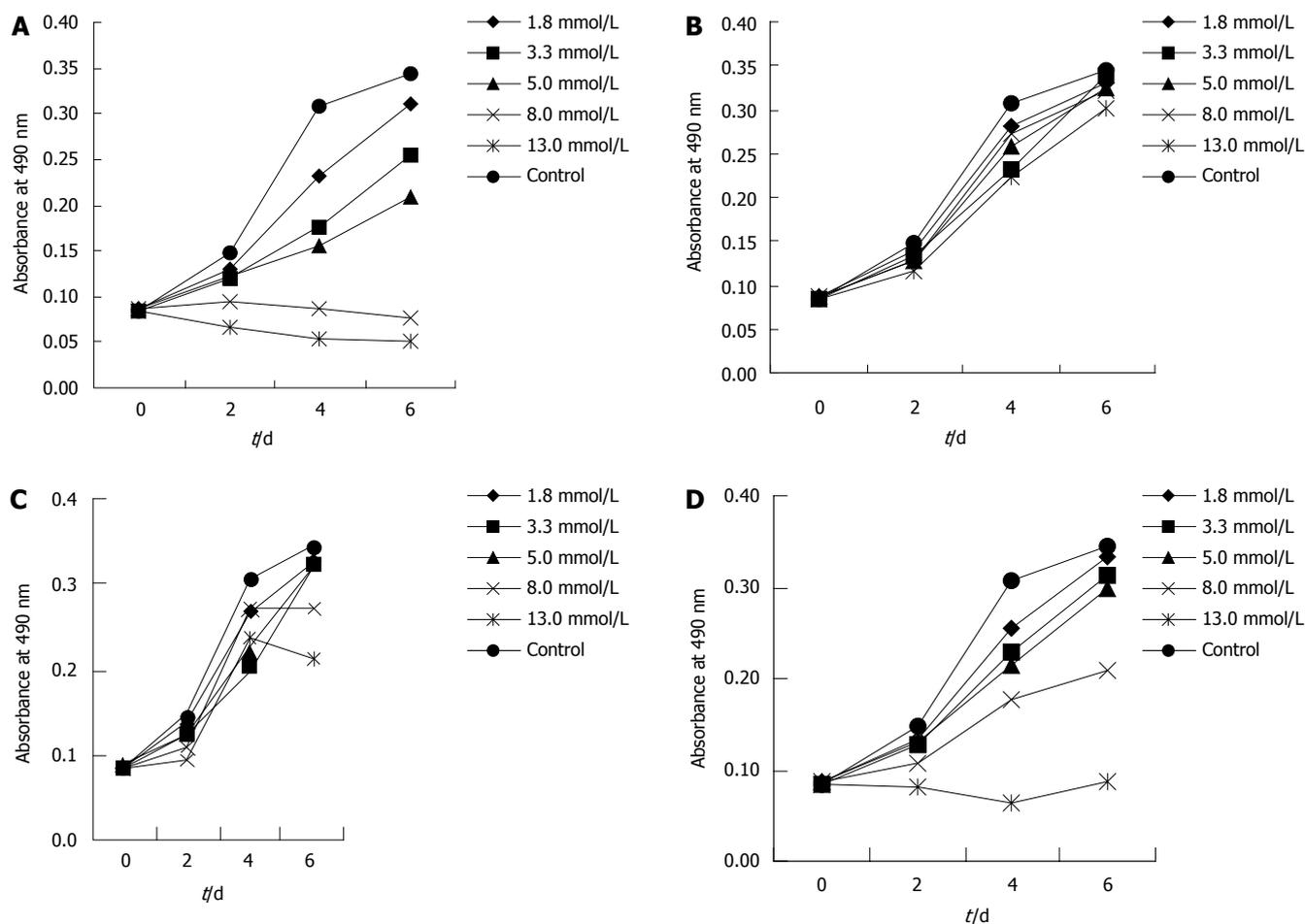


Figure 1 MTT assay showing the effect of IP₆ on the growth of HT-29 cells after treated for 0 (A), 6 (B), 12 (C), 24 h (D).

Table 2 P21 expression in IP₆-treated HT-29 cells (mean ± SD)

Concentration of IP ₆ (mmol/L)	Absorbance value
Control	0.4868 ± 0.0486
1.8	0.6011 ± 0.0152
3.3	0.5138 ± 0.0336
5.0	0.6032 ± 0.0105
8.0	0.6078 ± 0.0066
13.0	0.5981 ± 0.0163

Table 3 PCNA expression in IP₆-treated HT-29 cells (mean ± SD)

Concentration of IP ₆ (mmol/L)	Absorbance value
Control	0.6407 ± 0.0096
1.8	0.6361 ± 0.0087
3.3	0.5904 ± 0.0302
5.0	0.4520 ± 0.0495
8.0	0.4788 ± 0.0357
13.0	0.5006 ± 0.0403

DISCUSSION

Uncontrolled proliferation is one of the most important characteristics of malignant cells due to the aberrations of cell cycle regulators such as mutation, activation or inactivation of genes. Identification of cell cycle regulator specificity of anti-tumor drugs is essential to understand the mechanisms of their action.

MTT assay in this study showed that the growth of HT-29 cells was inhibited after continuous IP₆ treatment for 2-6 d ($P < 0.05$). The effect enhanced with increasing IP₆ concentration and prolonged treatment time, suggesting that the inhibition effects of IP₆ are dose- and time-dependent.

To confirm our data we used another proliferating marker, proliferating cell nuclear antigen (PCNA) which is

essential for both DNA replication and repair^[26]. During DNA replication, PCNA forms a ring structure clamping the synthesized DNA to the DNA polymerases δ and ϵ to ensure continuation of the replication process^[27,28]. In case of DNA damage, PCNA binds to the over-expressed P21^{wafl/cip1} leading to inhibition of PCNA-dependent replication, but it does not affect the DNA repair function attained by PCNA^[29]. Thus, PCNA is expressed in both cycling and non-cycling cells^[30]. Immunocytochemistry in this study showed that IP₆-treated cells reduced PCNA expression compared with control cells ($P < 0.05$), although the dose-dependent inhibition was not obvious, which was in agreement to the low proliferation rate observed in MTT assay, indicating that IP₆ inhibits proliferation of HT-29 cells.

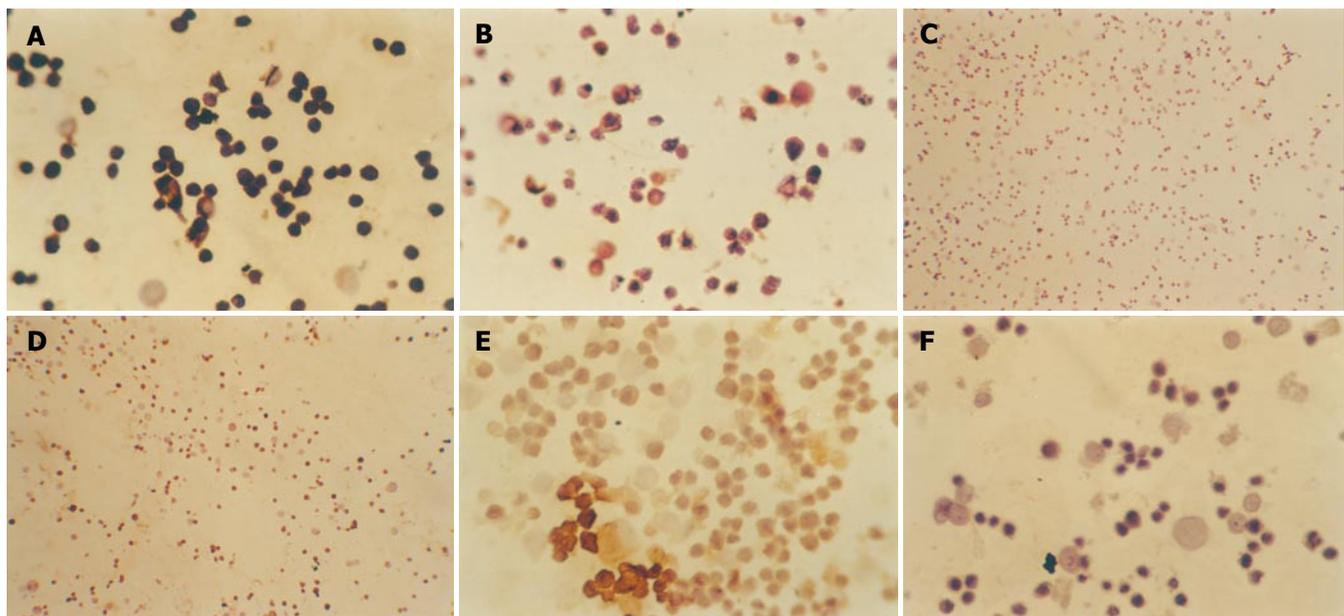


Figure 2 Expression of p53 (A, B), p21 (C, D) and PCNA (E, F) in untreated control and IP₆-treated cells. The slides were counterstained with hematoxylin ($\times 400$, $\times 100$, $\times 400$, respectively).

Table 4 Absorbance values assayed by MTT after continuous treatment with IP₆ (mean \pm SD)

IP ₆ (mmol/L)	Time (d)			
	0	2	4	6
Control	0.0847 \pm 0.0021	0.1487 \pm 0.0031	0.3083 \pm 0.0240	0.3450 \pm 0.0056
1.8	0.0857 \pm 0.0045	0.1293 \pm 0.0049	0.2320 \pm 0.0155	0.3097 \pm 0.0180
3.3	0.0837 \pm 0.0042	0.1203 \pm 0.0075	0.1770 \pm 0.0227	0.2537 \pm 0.0300
5.0	0.0873 \pm 0.0057	0.1233 \pm 0.0029	0.1547 \pm 0.0042	0.2077 \pm 0.0153
8.0	0.0857 \pm 0.0021	0.0940 \pm 0.0053	0.0873 \pm 0.0032	0.0757 \pm 0.0025
13.0	0.0847 \pm 0.0032	0.0660 \pm 0.0010	0.0530 \pm 0.0026	0.0510 \pm 0.0036

Table 5 Absorbance values assayed by MTT after IP₆ treatment for 6 h (mean \pm SD)

IP ₆ (mmol/L)	Time (d)			
	0	2	4	6
Control	0.0847 \pm 0.0021	0.1487 \pm 0.0031	0.3083 \pm 0.0240	0.3450 \pm 0.0056
1.8	0.0857 \pm 0.0045	0.1397 \pm 0.0023	0.2823 \pm 0.0344	0.3303 \pm 0.0080
3.3	0.0837 \pm 0.0042	0.1333 \pm 0.0015	0.2317 \pm 0.0192	0.3383 \pm 0.0025
5.0	0.0873 \pm 0.0057	0.1277 \pm 0.0025	0.2593 \pm 0.0131	0.3240 \pm 0.0082
8.0	0.0857 \pm 0.0021	0.0940 \pm 0.0053	0.0873 \pm 0.0032	0.0757 \pm 0.0025
13.0	0.0847 \pm 0.0032	0.1157 \pm 0.0035	0.2237 \pm 0.0215	0.3020 \pm 0.0080

Table 6 Absorbance values assayed by MTT after IP₆ treatment for 12 h (mean \pm SD)

IP ₆ (mmol/L)	Time (d)			
	0	2	4	6
Control	0.0847 \pm 0.0021	0.1487 \pm 0.0031	0.3083 \pm 0.0240	0.3450 \pm 0.0056
1.8	0.0857 \pm 0.0045	0.1383 \pm 0.0065	0.2660 \pm 0.0056	0.3267 \pm 0.0050
3.3	0.0837 \pm 0.0042	0.1230 \pm 0.0043	0.1990 \pm 0.0554	0.3220 \pm 0.0105
5.0	0.0873 \pm 0.0057	0.1250 \pm 0.0017	0.2290 \pm 0.0350	0.3217 \pm 0.0076
8.0	0.0857 \pm 0.0021	0.1103 \pm 0.0038	0.2693 \pm 0.0711	0.2723 \pm 0.0025
13.0	0.0847 \pm 0.0032	0.0957 \pm 0.0035	0.2363 \pm 0.0299	0.2107 \pm 0.0135

Table 7 Absorbance values assayed by MTT after IP₆ treatment for 24 h (mean \pm SD)

IP ₆ (mmol/L)	Time (d)			
	0	2	4	6
Control	0.0847 \pm 0.0021	0.1487 \pm 0.0031	0.3083 \pm 0.0240	0.3450 \pm 0.0056
1.8	0.0857 \pm 0.0045	0.1323 \pm 0.0040	0.2547 \pm 0.0153	0.3330 \pm 0.0122
3.3	0.0837 \pm 0.0042	0.1277 \pm 0.0099	0.2300 \pm 0.0161	0.3123 \pm 0.0115
5.0	0.0873 \pm 0.0057	0.1317 \pm 0.0032	0.2137 \pm 0.0168	0.2990 \pm 0.0190
8.0	0.0857 \pm 0.0021	0.1067 \pm 0.0021	0.1757 \pm 0.0116	0.2093 \pm 0.0105
13.0	0.0847 \pm 0.0032	0.0807 \pm 0.0021	0.0650 \pm 0.0036	0.0857 \pm 0.0051

Since IP₆ inhibits cell growth, we studied the regulators of cell cycle. P53, a tumor suppressor protein, is a nuclear transcription factor that controls cell cycle progression^[31,32], and plays a role in G1/S check point of cell cycle allowing the repair of damaged DNA^[33,34]. Mutations and deletions of the tumor suppressor gene p53 have been identified in about 50% of colorectal carcinomas and are associated with poor prognosis due to its weaker ability to inhibit cell proliferation. The half-life of wild-type P53 is very short and difficult to detect, while the mutant P53 protein has a much longer half life and can be examined by conventional immunohistochemical technology^[35]. Rodrigues NR *et al*^[36]

showed that over-expression of p53 is synonymous with mutation and HT-29 cells have mutations in codon 273 of the p53 gene, so HT-29 cells overproduce mutant p53 antigen. In our study, the immunocytochemical results showed that in IP₆-treated cells the abnormal expression of P53 protein decreased compared to control ($P < 0.05$), indicating that IP₆ reduces the expression of mutant P53 protein. It was reported that treatment of HT-29 cells with IP₆ increases the level of wild-type P53^[37]. In the present study, P53 polyclonal antibody was not specified for wild P53 but responded to many antigenic determinants, including mutant P53, indicating that IP₆ up-regulates

the expression of wild-type P53 and down-regulates the expression of mutant P53 to control cell cycle check-point and prevent progression of cells to the DNA synthesis phase (S phase) of the cell cycle. But the exact mechanism by which IP₆ affects p53 is not clear and needs further study.

P21^{waf1/cip1} is an inhibitor of cyclin dependent kinases (CDKs) that are required for the cells to enter the S-phase of the cell cycle^[38]. The gene encoding P21^{waf1/cip1} is transcriptionally regulated by the protein product of the gene p53. Over-expression of P21^{waf1/cip1} is growth inhibitory, possibly by inhibiting the activity of cyclin/CDK complex^[39] which binds to the C-terminal domain of PCNA. The resulting P21-PCNA complex blocks the ability of PCNA to process DNA polymerase in DNA replication. Thus P21^{waf1/cip1} may act as a tumor suppressor because of its role in growth control^[39,40]. In the present study, the expression of P21 was increased after IP₆ treatment for 2 d ($P < 0.05$). After counterstaining with hematoxylin, untreated cells were stained purple while IP₆-treated cells were stained yellow, indicating that expression of P21 is higher in IP₆-treated cells. High-expression of P21^{waf1/cip1} leads to decreased nuclear expression of PCNA, which is in agreement with our results.

In summary, IP₆ remarkably inhibits proliferation of HT-29 human colon carcinoma cell line. IP₆ exerts its inhibitory effect in part by affecting special cell cycle regulators and reduces over-expression of mutant P53 and stimulates expression of wild-type P53 and P21^{waf1/cip1}. P21^{waf1/cip1} binds to PCNA, thus preventing PCNA-dependent cellular proliferation. In our immunocytochemical experiments, cells grew very slowly and were not adhered in media with high IP₆ dose, fell off and died very soon. The effect of 13.0 mmol/L IP₆ on expression of genes was less than that of 8.0 mmol/L IP₆, partly due to the rapid death of cells in 13.0 mmol/L IP₆, indicating that that IP₆ has no significant effect on the expression of genes. Furthermore, neither significant dose-dependent effect of IP₆ was observed on the expressions of cell cycle regulators nor obvious correlation among these indexes was found, possibly owing to the short period of IP₆ treatment (only 2 d), suggesting that the effects of IP₆ on gene expressions are relatively weak.

The present study is merely a preliminary investigation of IP₆ on colon cancer. The results are also limited although the effects of IP₆ can be seen. Additional research is needed to explore the mechanisms of IP₆ in cell proliferation and differentiation, apoptosis, and potential therapeutic value of IP₆.

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Amplification of chromosome 21q22.3 harboring trefoil factor family genes in liver fluke related cholangiocarcinoma is associated with poor prognosis

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prognosis, whereas patients who had deletion showed favorable prognosis (mean: 51.7 wk vs 124.82 wk, $P = 0.012$). Multivariate Cox regression analysis revealed that amplification of D21S1893, D21S1890 and *TFF*, blood vessel invasion, and staging were associated with poor prognosis.

CONCLUSION: D21S1893-D21S1890 region may harbor candidate genes especially *TFF* and serine protease family, which might be involved in tumor invasion and metastasis contributing to poor survival. The amplification in this region may be used as a prognostic marker in the treatment of CCA patients.

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Key words: Cholangiocarcinoma; Amplification on chromosome 21; Trefoil factor family; Quantitative PCR; Liver fluke

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Abstract

AIM: To determine allelic imbalance on chromosomal region 21q22-qter including trefoil factor family genes (*TFF*) in cholangiocarcinoma (CCA) patients and analyze the correlation between allelic imbalances and clinicopathological parameters.

METHODS: Quantitative PCR amplification was performed on four microsatellite markers and trefoil factor family genes (*TFF1*, *TFF2*, and *TFF3*) using a standard curve and SYBR Green I dye method. The relative copy number was determined by DNA copy number of tested locus to reference locus. The relative copy number was interpreted as deletion or amplification by comparison with normal reference range. Associations between allelic imbalance and clinicopathological parameters of CCA patients were evaluated by χ^2 -tests. Kaplan-Meier method was used to analyze survival.

RESULTS: The frequencies of amplification at D21S1890, D21S1893, and *TFF3* were 32.5%, 30.0%, and 28.7%, respectively. Patients who had amplification at regions covering D21S1893, D21S1890, and *TFF* showed poor

INTRODUCTION

Cholangiocarcinoma (CCA) or intrahepatic bile duct cancer (ICC) is a malignant tumor in the biliary tree peripheral to the bifurcation of the right and left hepatic duct^[1,2]. Incidence rates of ICC vary substantially worldwide, reflecting the distribution of local geographic risk factors, in addition to genetic differences among various populations. In Western countries, the disease is rare, however, it is highly frequent in Southeast Asia, especially in Khon Kaen, Northeast Thailand. Truncated age-standardized incidence of CCA at ages > 35 years varied by three fold between districts, from 93.8 to 317.6 per 100 000 population^[3]. In Western countries, primary sclerosing cholangitis is the commonest known predisposing condition for this cancer. Eight percent to 40% of CCA have been reported in patients with primary

Table 1 Locations and sequences of microsatellite markers, trefoil factor family genes and reference loci

Primer name	Chromosome	Product size (bp)	Forward primer	Reverse primer
D21S1253	21q21.3	174-190	GAAGAATCTCCGAACCAGG	AAGACCAGTGTATTTCAGAGCC
D21S1255	21q22.2	112-126	AGCTCTTTATTTTGCCACATAG	CTGCATGTTTGCCTGG
D21S1893	21q22.2	111-119	GTATGCACACCACACGG	TAAACAAAATCCGCCACG
D21S1890	21q22.3	143-173	GGTCTGACCACAGATTTCC	AAAAAACTCTGAACGATTAAGG
Trefoil factor family 1	21q22.3	219	CAGGGATCTGCCTGCATC	ATCGATCTCTTTAATTTTAGGCC
Trefoil factor family 2	21q22.3	123	GAAGAATCTCCGAACCAGG	GTCACACTCAAAAACTAGAGG
Trefoil factor family 3	21q22.3	129	CAGGCACGTTCATCTCAGC	TATTCGTTAAGACATCAGGCTCC
<i>β-actin</i>	7p15	375	TCACCCACACTGTGCCATCTACGA	CAGCGGAACCGTCTATGCCAATGG
<i>GAPDH</i> ¹	12p13	250	ACAGTCCATGCCATCACTGCC	GCCTGCTCACCACCTTCTTG

¹Glyceraldehyde-3-phosphate dehydrogenase.

sclerosing cholangitis^[4]. Several clinical studies and animal model experimental studies suggest that the interaction between chemical carcinogens, especially nitrosamines and *Opisthorcis viverrini* infestation may play an important role in the development of CCA in Thailand^[5-7]. Thus, either the chemical carcinogen nitrosamine or liver fluke infection alone does not produce cancer. Food derived exogenous and *in situ* nitrosamine formation may lead to DNA alkylation and also deamination in predisposed and inflamed tissues. Furthermore, chronic irritation caused by the fluke results in hyperplasia and adenomatous change of bile duct epithelium^[6]. The DNA damaged biliary epithelium may then be transformed to malignant CCA^[7-9]. To date, the molecular basis of carcinogenesis and pathogenesis of cholangiocarcinoma is still unclear.

Allelic imbalance at specific genomic loci is an important step in the molecular genetic analysis of human cancers. Allelic imbalance at chromosome 21, especially region 21q22-qter, was found in several types of human cancers such as gastric cancer, breast cancer, ovarian clear cell adenocarcinoma, and primary colorectal cancer^[10-13]. Furthermore, chromosome 21q22.3 harbors a cluster of trefoil factor family (*TFF*) genes consisting of *TFF1*, *TFF2*, and *TFF3*^[14]. *TFF* functions include mucus stabilization and stimulation of normal epithelial cell restitution during wound repair through motogenic and antiapoptotic activities. However, *TFF* peptides are overexpressed in several human solid tumors such as prostate, esophagus, breast, and pancreas and also function as tumor progression factor^[15-18]. Prolonged inflammation caused by parasitic infection frequently occurs in liver fluke related CCA. *TFF* and its neighborhood located at 21q22 may be involved in tumor development and progression. Moreover, our data on comparative genomic hybridization (CGH) in CCA showed the alteration of DNA copy number at 21q22-qter at 28%.

Taken these data together, the chromosomal region 21q22-qter may harbor candidate genes, which are involved in carcinogenesis and pathogenesis of CCA. Therefore, this study attempted to determine allelic imbalance on chromosomal region 21q22-qter including *TFF* genes to define affected sites for candidate genes which are involved in molecular carcinogenesis and pathogenesis of CCA. The associations between allelic imbalance and clinicopathological parameters were also determined.

MATERIALS AND METHODS

Samples and DNA preparation

This project was approved by the Ethical Committee of Khon Kaen University. Informed consents were obtained from patients who were willing to participate in the project. Frozen liver tissues were obtained from 80 CCA patients undergoing surgical resection at Srinagarind Hospital, Faculty of Medicine, Khon Kaen University, Thailand. CCA cases were diagnosed by physicians according to clinical finding, laboratory investigation and histological examination. Neural, blood vessel and lymphatic invasion were assessed by standard method^[19]. The clinicopathological data such as age, gender, histological type, and TNM stage^[20] were evaluated by reviewing the medical charts and pathology records. DNA was prepared from frozen liver tissues containing 80% of tumor cells by using a PuregeneTM DNA purification system (Gentra System, USA) according to manufacturer's instructions. In addition, DNA was prepared from placental tissue collected from a normal labor (postpartum) woman and used for setting a standard curve. Normal leukocyte DNA derived from 50 healthy donors was prepared into 14 pooled normal DNA and generated for normal reference range.

Quantitative PCR assay

Quantitative PCR amplification was performed on a Rotor Gene 2000 Real-time Amplification (Corbett Research, Australia) using four microsatellite markers (telomere-D21S1890-D21S1893-D21S1255-D21S1253-centromere) and trefoil factor family genes (*TFF1*, *TFF2*, and *TFF3*) covering chromosomal region 21q21-qter. Reference primers were chosen in the region of the housekeeping genes that usually are not altered alteration in CCA, *β-actin* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Primer sequences were obtained from the Genome Data Base. Locations of selected oligonucleotides and their sequences are shown in Table 1. PCR reaction was performed in a 25 μ L reaction mixture containing 50 mmol/L KCl, 10 mmol/L Tris HCl pH 8.3, 100 μ mol/L each of deoxynucleoside triphosphate (dNTP), 2.5 mmol/L MgCl₂ or 3.0 mmol/L (D21S1253, *TFF1*, and *β-actin*), 1.25 μ L SYBR[®] Green I dye solution (Amresco, USA) (1:10 000 in DMSO), and 10 μ mol/L or 5 μ mol/L (D21S1253 and

D21S1255) or 15 μmol/L (D21S1890) of each primer with 1.5 units or 2.0 units (D21S1253, D21S1255, and *GAPDH*) of *Taq* DNA polymerase. The PCR was performed at 95°C for 5 min followed by 95°C for 15 s, 45°C -60°C for 15 s and 72°C for 15 s for 35 cycles with an additional cycle of 72°C for 10 min.

Quantitative PCR amplification was performed using a standard curve and SYBR Green I dye method as described previously^[21]. The standard curve for each primer was generated using serial dilutions of placental DNA. The standard curve was constructed in each PCR run and the copy numbers of genes in each sample were interpolated using these standard curves. Placental DNA with known concentration was used for precision control. A coefficient of variation (CV) of each sample was determined based on triplicate test. The sample with a CV higher than 15% was re-tested. DNA copy number of each locus was calculated based on triplicate determination and duplicate PCR run.

Analysis of allelic imbalance

The relative copy number was determined by DNA copy number of tested locus to DNA copy number of reference locus. The DNA copy numbers of reference loci consisting of *β-actin* and *GAPDH* were averaged before calculation. The normal reference range was generated from the relative copy numbers of 14 pooled normal leukocyte DNA of 7 markers (*n* = 98). If the relative copy number of sample calculated differed significantly from normal reference range (mean ± 2SD), the sample was verified as loss or gain. The relative copy number was interpreted as loss when the ratio was less than mean -2SD of normal reference range. On the other hand, the relative copy number was interpreted as gain when the ratio was more than mean + 2SD of normal reference range.

Statistical analysis

Associations between allelic imbalance and clinicopathological parameters of 80 CCA patients were evaluated by means of the χ^2 -tests. Survival curves for patients with allelic imbalance versus those without were calculated using the Kaplan-Meier method. Only 69 cases were available for follow-up. Six patients were lost for follow-up and five cases were perioperative death (patients who died within 4 wk after surgery). Differences in survival between these two groups were assessed by the log-rank test. Cox proportional hazards model was used in univariate and multivariate analysis. *P* < 0.05 was considered statistically significant.

RESULTS

Allelic imbalance on chromosomal region 21q22-qter

The normal reference range generated from relative copy numbers of pooled normal leukocyte DNA was 0.54-1.34 with 95% confidence interval (mean ± 2SD). Allelic imbalance of 7 loci showed percentages of amplification at D21S1890 (32.5%), D21S1893 (30.0%), *TFF3* (28.8%), D21S1253 (26.3%), D21S1255 (23.8%), *TFF1* (22.5%), and *TFF2* (7.5%) and of deletion at *TFF3* (3.8%), D21S1255 (2.5%), *TFF2* (2.5%), and D21S1890 (1.3%).

No	D21S1890	TFF1	TFF2	TFF3	D21S1893	D21S1255	D21S1253
1							
2							
3							
4							
5							
6							
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Figure 1 Fine mapping of allelic imbalance on chromosomal region 21q22-qter. D21S1890 is located at telomeric end while D21S1253 is located toward centromeric end. There are two common amplification regions at D21S1890 and the region between D21S1893 and *TFF3*.

The relative copy number of amplification of these loci ranged between 1.35-4.24 and of deletion between 0.39-0.52. Fine mapping of these regions is shown in Figure 1. Two regions of common amplification were D21S1890 and the region between D21S1893 and *TFF3*.

Associations between allelic imbalance and clinicopathological parameters of patients

The associations between allelic imbalance and clinicopathological parameters of patients were analyzed. The result showed no differences in age, sex, histological type, invasion (blood vessel, lymphatic, and nerve), and survival time between patients with and without

Table 2 Clinicopathological parameters of patients with and without allelic imbalance

Clinical parameters	n	Gene/microsatellite makers D21S1893, D21S1890 and TFF			P value
		Normal n (%)	Amplification n (%)	Deletion n (%)	
Age (yr)	80				
≤ 54	39	18 (46)	20 (51)	1 (3)	NS
> 54	41	15 (36)	22 (54)	4 (10)	
Gender	80				
Male	57	22 (39)	32 (56)	3 (5)	NS
Female	23	11 (48)	10 (43)	2 (9)	
Histological type	80				
Non-Papillary adenocarcinoma	58	27 (46)	30 (52)	1 (2)	0.023
Papillary adenocarcinoma	22	6 (27)	12 (55)	4 (18)	
Staging	80				
II & III	10	5 (50)	5 (50)	-	NS
IVA & IVB	70	28 (40)	37 (52.8)	5 (7.2)	
Blood vessel invasion	79				
Non-invasion	28	8 (29)	16 (57)	4 (14)	NS
Invasion	51	24 (47)	26 (51)	1 (2)	
Lymphatic invasion	79				
Non-invasion	15	4 (27)	11 (73)	-	NS
Invasion	64	28 (43)	31 (49)	5 (8)	
Nerve invasion	79				
Non-invasion	36	14 (39)	19 (53)	3 (8)	NS
Invasion	43	18 (42)	23 (54)	2 (4)	
Survival time (wk)	69				0.012
Mean		54.12	51.7	124.82	
Median		49	27.4	84.28	
Minimum-maximum		7.14-119.28	4.85-242.85	67.71-143.85	

allelic imbalance in almost all loci ($P > 0.050$). However, significant difference was observed in histological type at loci covering D21S1893 through D21S1890 including *TFF* ($P = 0.023$). Deletion at D21S1893, D21S1890, and *TFF* found in a papillary type was higher than that in a non-papillary one (Table 2). Furthermore, patients who had deletion in this region tended to show non-invasion of blood vessel ($P = 0.052$) (Table 2). Kaplan-Meier survival curves demonstrated that patients who had amplification at D21S1893, D21S1890, and *TFF* showed poor prognosis, whereas patients who had deletion at D21S1893, D21S1890, and *TFF* showed favorable prognosis ($P = 0.012$) (Figure 2). Multivariate analysis showed significant effects of amplification at D21S1893, D21S1890, and *TFF*, blood vessel invasion, and staging on prognosis (Table 3).

DISCUSSION

Many techniques have been used to detect genetic alterations. The real-time qPCR technique is an alternative method to determine allelic imbalance because most allelic imbalances also result in changes of relative copy numbers. In our study, allelic imbalance determined by qPCR using SYBR Green I system showed CV less than 15%, suggesting good consistency and reliability^[21]. The house keeping genes, *β-actin* and *GAPDH*, were used as reference loci in

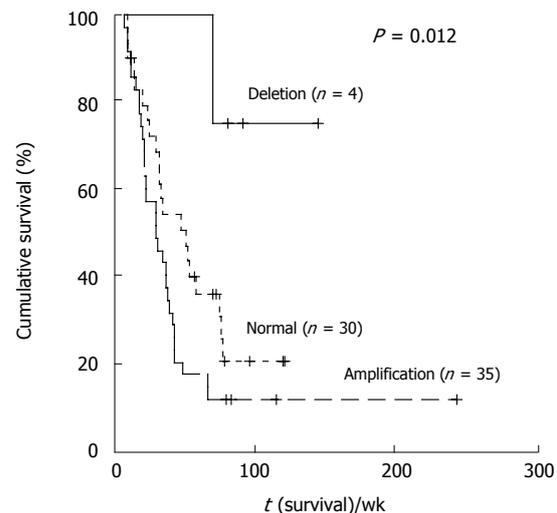


Figure 2 The association between survival time and allelic imbalance on loci D21S1893, D21S1890 and *TFF* was analyzed by Kaplan-Meier. The patients who had amplification at D21S1893, D21S1890 and *TFF* showed poor prognosis, whereas patients who had deletion at D21S1893, D21S1890 and *TFF* showed favorable prognosis.

our study. To assess the validity of these reference genes, we determined the copy number ratios of *β-actin/GAPDH* in 14 pooled normal leukocytes and 80 CCA samples. The observed ratios measured from normal DNA ($1.01 \pm 0.14SD$) and tumor DNA ($1.06 \pm 0.15SD$) were similar ($P = 0.271$) and both were significantly equal to 1, thus, confirming their validity as appropriate reference genes in our work.

Our finding of allelic imbalance on chromosomal region 21q22-qter in CCA patients showed predominant amplifications at markers D21S1890 (32.5%) and D21S1893 (30%). This region has length about 3.5 Mb in physical map distance and contains about 25 identified genes. Trefoil factor family (*TFF1*, *TFF2*, and *TFF3*) and serine protease family (*TMPRSS2* and *TMPRSS3*) are candidate genes located in this region and have a potential tumor progression activity. In this study, all CCA tissues were obtained from patients who were residents of north-eastern region of Thailand where liver fluke infection remains highly endemic. Increased gene amplification at chromosome 21q22.3 especially *TFF* genes in liver fluke related CCA may result from healing process of inflamed tissues. Normally, TFF peptides are involved in the normal mucosal defense and epithelial restitution in cell injury. However, in chronic inflammation, the overproduction of TFF peptides may result in tumor progression. TFF peptides might exert beneficial effects during the early step of mucosal injury and inflammation and subsequently undesirable effects during chronic inflammation and neoplastic progression. TFF peptides function as a tumor progression factor by increasing cell scattering, invasion, survival and proangiogenesis^[22-25]. *TFF3* stimulates cell motility by inducing a rapid phosphorylation of β -catenin, which is associated with perturbation of the functional integrity of E-cadherin/catenin system. The promotion of cell motility in association with epidermal growth factor receptor (EGFR) leads to the enhancement of tumor cell invasion and metastasis^[26,27]. However, exogenous TFF peptides

Table 3 Univariate and multivariate analysis of overall survival in cholangiocarcinoma

Variable	n	Univariate		Multivariate	
		Relative risk (95%CI)	P value	Relative risk (95%CI)	P value
Age (yr)	69	0.831 (0.483-1.430)	NS		
≤ 54	34				
> 54	35				
Gender	69	0.443 (0.231-0.849)	0.014	0.628 (0.312-1.263)	NS
Male	49				
Famale	20				
Histological type	69	0.622 (0.332-1.167)	NS		
Non-Papillary adenocarcinoma	50				
Papillary adenocarcinoma	19				
Staging	69	2.265 (0.899-5.708)	0.083	3.320 (1.270-8.681)	0.014
II & III	9				
IVA & IVB	60				
Blood vessel invasion	69	2.108 (1.139-3.902)	0.018	2.183 (1.088-4.382)	0.028
Non-invasion	23				
Invasion	46				
Lymphatic invasion	69	0.981 (0.479-2.012)	NS		
Non-invasion	12				
Invasion	57				
Nerve invasion	69	1.129 (0.654-1.950)	NS		
Non-invasion	32				
Invasion	37				
D21S1893, D21S1890, and TFF	69		0.026		0.002
Normal	30	Reference		Reference	
Amplification	35	1.707 (0.974-2.991)	NS	2.473 (1.342-4.557)	0.004
Deletion	4	0.187 (0.025-1.394)	NS	0.224 (0.029-1.701)	NS

alone are not sufficient to induce the invasive phenotype in premalignant human colonic adenoma cells PC/AA/C1 and kidney MDCK epithelial cells, but require the priming and permissive action of src and RhoA to exert their proinvasive activity^[22]. These observations suggest that trefoil peptides elicit a coordinated cellular response enabling cell migration without triggering the programmed cell death response usually precipitated by cell detachment from a stationary anchored state. TFF1 protects cells from anoikis, chemical-, or Bad-induced apoptosis by reducing caspase-3, caspase-6, caspase-8, and caspase-9 activities^[25]. TFF3 may act as anti-apoptosis by preventing p53-dependent and p53-independent apoptosis pathways^[28]. The anti-apoptotic effects of TFF3 are associated with activation of the PI3K-Akt signaling pathway. Thus, TFF functions as an anti-apoptosis factor, resulting in an increase in number of survived cancer cells through proliferation *via* Ras/MEK/MAP kinase signaling transduction pathway. Angiogenic activity of TFF is comparable to that induced by vascular endothelial growth factor (VEGF), leptin, and transforming growth factor- α . Stimulation of angiogenesis by TFF1 in the chick chorioallantoic membrane (CAM) assay was COX-2- and EGFR-dependent, but independent

of the VEGF receptor KDR/flk-1 and the thromboxane A2 receptor (TXA-2-R). These results implicate a role of TFF in the formation of new blood vessels during normal and pathophysiological processes linked to wound healing, inflammation, and cancer progression^[23].

Transmembrane protease, serine 2 (*TMPRSS2*) and transmembrane protease, serine 3 (*TMPRSS3*) are members of serine protease family. Proteases have been increasingly recognized as important factors in the pathophysiology of tumorous diseases. Members of the endopeptidases, such as serine protease family, mediate the proteolytic degradation of the extracellular matrix, which is an indispensable step in tumor invasion and metastasis^[29]. *TMPRSS2* was highly expressed in prostate cancer and correlated with the metastatic potential and involved in microvascular endothelial cell reorganization and capillary morphogenesis^[30,31]. *TMPRSS3* is strongly expressed in a subset of pancreatic cancer and various other cancer tissues, and its expression correlates with the metastatic potential of the clonal SUI-2 pancreatic cancer cell lines^[32]. The data suggested that both *TMPRSS2* and *TMPRSS3* may be important for the processes involved in metastasis, invasion, and angiogenesis in tumor cells. Our study showed high amplifications of markers D21S1893 and D21S1890, suggesting that existence of candidate genes might be involved in pathogenesis of CCA. The data regarding the involvement of serine protease and *TFF* in aggressive feature and metastasis supported our finding that amplifications of candidate genes at regions D21S1893, D21S1890, and *TFF* were found in poor prognostic CCA patients. Clinical data of CCA patients supported our hypothesis that TFF stimulated cell motility via E-cadherin/catenin and APC complexes and promoted tumor cell survival by anti-apoptosis, while serine protease mediated vascular endothelial invasion and angiogenesis leading to poor survival in CCA patients. Although the differences in genetic alterations between liver fluke related and non-liver fluke related CCA have been observed previously^[33,34], the conclusion regarding the difference between these two groups in allelic imbalance on chromosome 21q22 cannot be drawn.

As far as we know, allelic imbalance on the chromosomal region 21q22-qter including *TFF* in CCA patients is first reported by our group. The protein expression and functions of *TMPRSS2*, *TMPRSS3*, and *TFF* related to cancer invasion and metastasis in liver fluke related CCA patients require further study. The application of allelic imbalance on D21S1893, D21S1890, and *TFF* may be of value as a prognostic marker and a selection for CCA patient treatment.

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Antioxidant role of heme oxygenase-1 in prehepatic portal hypertensive rats

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totally prevented this effect.

CONCLUSION: These results suggest a beneficial role of HO-1 overexpression in prehepatic portal hypertensive rats.

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Key words: Heme oxygenase-1; Portal hypertensive rats; Liver oxidative stress

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Abstract

AIM: To study the effect of bilirubin on the oxidative liver status and the activity and expression of heme oxygenase-1 (HO-1) in rat liver injury induced by prehepatic portal hypertension.

METHODS: Wistar male rats, weighing 200-250 g, were divided at random into two groups: one group with prehepatic portal hypertension (PH) induced by regulated prehepatic portal vein ligation (PPVL) and the other group corresponded to sham operated rats. Portal pressure, oxidative stress parameters, antioxidant enzymes, HO-1 activity and expression and hepatic sinusoidal vasodilatation were measured.

RESULTS: In PPVL rats oxidative stress was evidenced by a marked increase in thiobarbituric acid reactive substances (TBARS) content and a decrease in reduced glutathione (GSH) levels. The activities of liver antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) were also diminished while activity and expression of HO-1 were enhanced. Administration of bilirubin (5 µmol/kg body weight) 24 h before the end of the experiment entirely prevented all these effects. Pretreatment with Sn-protoporphyrin IX (Sn-PPIX) (100 µg/kg body weight, i.p.), a potent inhibitor of HO, completely abolished the oxidative stress and provoked a slight decrease in liver GSH levels as well as an increase in lipid peroxidation. Besides, carbon monoxide, another heme catabolic product, induced a significant increase in sinusoidal hepatic areas in PPVL group. Pretreatment of PPVL rats with Sn-PPIX

INTRODUCTION

Portal hypertension (PH) constitutes a major complication in chronic liver diseases including cirrhosis. It is associated with a hyperdynamic splanchnic circulation in response to the resistance of portal blood flow to reach the liver. This complication is associated with collateralization of the portal system, leading to the development of varix at various locations in the upper gastrointestinal tract^[1].

Oxidative stress is the result of excessive generation of reactive oxygen species (ROS), depletion of intracellular antioxidant defences or a combination of both, leading to an imbalance in the redox status of the cell. Reactive oxygen species induce cell, tissue or organ damage and ROS have been proposed as a major factor responsible for several diseases, and have been implicated in portal hypertension^[2].

ROS occur in tissues and may damage DNA, proteins, carbohydrates, and lipids. These potentially deleterious reactions are controlled by a system of antioxidant defences, which eliminate pro-oxidants and scavenge free radicals. Various intracellular compounds such as glutathione, and antioxidant enzymes including catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px)^[3] provide protection against oxidation. Heme oxygenase (HO) is the key microsomal enzyme in heme degradation to carbon monoxide (CO), iron and biliverdin, the latter being converted into bilirubin by the cytosolic biliverdin reductase^[4,5]. Heme oxygenase, the rate limiting enzyme in heme degradation pathway, is induced in animal tissues, particularly in liver by many factors including its own

substrate heme, several heme-proteins, heavy metals, UVA radiation, hypoxia, hyperoxia and others^[6-9]. Induction of HO is entirely prevented by administration of several antioxidants such as α -tocopherol and allopurinol^[9]. In recent years good evidence has been accumulated showing that bilirubin can act as a highly effective antioxidant and free radical scavenger, and it has been proposed that it can play a physiological and key role as an endogenous protective agent against oxidant mediated injury^[10-14].

Antioxidant effects suggest that oxidant species play a major part in the induction of HO either directly or by GSH depletion^[9,10,15]. An increase in HO activity will enhance bilirubin formation and because unconjugated bilirubin is an efficient scavenger of ROS, its increase would be the cellular response to oxidative stress^[10,12-14]. Besides, similar to nitric oxide, CO derived from heme oxygenase reaction also acts as a neurotransmitter and regulator of vascular tone^[16].

Of the two known mammal liver HO isoenzymes, HO-1 and HO-2^[17], only HO-1 is inducible, herewith we will only refer to HO-1.

The aim of this work was to investigate the effect of HO-1 overexpression in rat livers with prehepatic portal hypertension.

MATERIALS AND METHODS

Animals and surgical procedures

Wistar male rats (200-250 g) were housed separately and acclimatised before use under conditions of controlled temperature ($25 \pm 2^\circ\text{C}$) and illumination (12 h light/dark cycle). Rats were fed with standard rat chow and water *ad libitum*. After 1 wk of acclimatisation, rats were randomised and separated into two groups: (1) Sham operated ($n = 24$) and (2) Prehepatic portal vein ligated rats ($n = 24$) (PPVL). Portal hypertension was induced by a calibrated portal vein stenosis according to the procedure of Chojkier *et al*^[18]. In brief, rats were anaesthetised with sodium pentobarbital (50 mg/kg body weight, i.p.) and then a midline abdominal incision was made. The portal vein was located and isolated from the surrounding tissues. A ligature of 3-0 silk was placed around the vein and snugly tied it to a 20 gauge blunt end needle placed along side the portal vein. The needle was subsequently removed to yield a calibrated stenosis of the portal vein. Sham rats underwent an identical procedure except that portal vein was isolated but not stenosed. Fourteen days after the operation rats of group 2 developed portal hypertension. Then, one day before the experiment, portal hypertensive and Sham rats were divided into three subgroups. One of each subgroup received one bolus injection of bilirubin (5 $\mu\text{mol/kg}$ body weight, i.p) or one bolus injection of Sn-protoporphyrin IX (Sn-PPIX; 100 $\mu\text{mol/kg}$ body weight, i.p). Then, six groups of animals were used ($n = 8$): Sham group (Sham), PPVL, Sham pretreated with bilirubin group (Sham + bilirubin), PPVL pretreated with bilirubin group (PPVL + bilirubin), sham pretreated with Sn-PPIX group (Sham + Sn-PPIX) and PPVL pretreated with Sn-PPIX group (PPVL + Sn-PPIX). Bilirubin and Sn-PPIX were prepared as follows: solutions in 0.1 mol/L NaOH were freshly prepared before administration, adjusted to pH 7.4 with

phosphate buffer and diluted with saline. In each subgroup portal pressure was measured immediately before the sacrifice. Fourteen days after the corresponding operation and before sacrifice, the rats were anaesthetised with sodium pentobarbital (50 mg/kg), intraperitoneally (i.p.). Portal pressure was measured through a needle placed in the splenic pulp, and maintained in place by cyanoacrylate gel. The needle was cannulated to a polyethylene catheter (50) filled with a heparinized saline solution (25 U/mL) and connected to a Statham Gould P23ID pressure transducer (Statham, Hato Rey, Puerto Rico) coupled to a Grass 79D polygraph (Grass Instruments, Quincy, MA). Animals were treated in accordance with guidelines established by the Animal Care and Use Committee of the Argentine Association of Specialists in Laboratory Animals (AADEALC), and were in accordance with the Guide to the Care and Use of Experimental Animals published by the Argentine Council on Animal Care.

Enzyme preparations and assays

Rats were anaesthetised with sodium pentobarbital (50 mg/kg body weight, i.p.) and killed 14 d after surgery. Livers were excised and perfused "in situ" with ice-cold saline solution (0.9% NaCl), then excised and homogenised in a Potter-Elvehjem homogenizer using different solutions. For heme oxygenase assay the homogenate was prepared using 4 vol of ice-cold 0.25 mol/L sucrose solution containing 1 mmol/L phenylmethyl sulfonyl fluoride, 0.2 mmol/L EDTA and 50 mmol/L potassium phosphate buffer (pH 7.4). Homogenates were centrifuged at 20000 g for 20 min and supernatant fractions centrifuged at 150000 g for 90 min. The microsomal pellet obtained was washed and resuspended in 20 mmol/L potassium phosphate buffer (pH 7.4), containing 135 mmol/L KCl, 1 mmol/L phenyl-methylsulfonyl fluoride and 0.2 mmol/L EDTA to a protein concentration of 10 mg/mL. Microsomal HO-1 was obtained as described elsewhere^[17]. The 150000 g supernatants obtained from the microsomal preparation were fractionated by addition of ammonium sulfate (AS), and the 40%-60% AS fraction dissolved in 10 mmol potassium phosphate buffer (pH 7.4) and dialyzed against the same buffer using this preparation as biliverdin reductase. Heme oxygenase activity was determined as described elsewhere^[10]. The standard incubation mixture in a final volume of 200 μL contained 10 μmol potassium phosphate buffer (pH 7.4), 60 nmol NADPH, 50 μL HO-1 (0.5 mg protein), 50 μL biliverdin reductase (0.42 mg protein), and 200 nmol hemin. Incubations were carried out at 37°C for 30 min. Activity was determined by measuring bilirubin formation, which was calculated as the difference in absorbance measured at 455 and 520 nm, employing an ϵ value of $50 \text{ mM}^{-1} \text{ cm}^{-1}$ ($\text{vis}_{\text{max}} 455 \text{ nm}$)^[11]. CAT, SOD and GSH-Px activities were determined spectrophotometrically in liver homogenates prepared in a medium consisting of 140 mmol/L KCl and 25 mmol/L potassium phosphate buffer (pH 7.4), and centrifuged at 600 g for 10 min. The supernatant, a suspension of preserved organelles, was used as homogenate. Catalase activity was determined by measuring the decrease in absorbance at 240 nm^[19] glutathione peroxidase activity following NADPH oxidation at 340 nm^[20], and superoxide dismutase

activity by inhibition of adrenochrome formation rate at 480 nm^[21]. One unit in the SOD assay is defined as the amount of enzymatic protein required to inhibit 50% of epinephrine auto-oxidation.

Lipid peroxidation

Lipid peroxidation in liver was determined by measuring the rate of production of thiobarbituric acid reactive substances (TBARS), expressed as malondialdehyde equivalents^[22]. One volume of homogenate was mixed with 0.5 volume TCA (15% w/v) and centrifuged at 2000 g for 10 min. The supernatant (1 mL) was mixed with 0.5 mL thiobarbituric acid (0.7% w/v) and boiled for 10 min. After cooling, sample absorbance was read spectrophotometrically at 535 nm. Malondialdehyde concentration was calculated using an ϵ value of $1.56 \times 10^5 \text{ M}^{-1}\text{cm}^{-1}$.

Endogenous hepatic GSH content

Total glutathione (GSH plus GSSG) was determined in liver homogenates after precipitation with 2% perchloric, and using yeast-glutathione reductase, 5, 5' dithio-bis-(2-nitrobenzoic acid) (DTNB) and NADPH and reading at 340 nm. GSSG was determined by the same method in the presence of 2-vinylpyridine. GSH was calculated from the difference between total glutathione and GSSG^[23].

Western-blot analysis of HO-1 expression

Samples of homogenate obtained for HO-1 activity assays were also analyzed by Western immunoblot technique as previously described^[24]. An amount of protein (50 μg) from homogenates of control and treated rats was run in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis using a 12% acylamide resolving gel (Mini Protean II System, BioRad, Hertz, UK). Separated proteins were transferred to nitrocellulose membranes and non-specific binding of antibodies was blocked with 3% non-fat dried milk in PBS, pH 7.4 for 1 h at room temperature. Membranes were then probed with polyclonal goat anti-HO-1 antibody (Santa Cruz, BioTech, California)(1:300 dilution in Tris-buffered saline, pH 7.4) overnight at 4°C. Immune complexes were detected using donkey anti goat secondary antibody (1:1500) (Santa Cruz, BioTech, California), and were visualized using ECL reagent (Amersham, Pharmacia). Intensity of bands was analyzed with Gel-Pro[®] analyzer 3.1 version, Media Cybernetics.

Microscopy and image analysis

Hepatic tissue was fixed in buffered formalin and stained with routine techniques (HE, Reticulin and Masson's trichromic) for light microscopy. For high resolution optic microscopy (HROM) tissue was fixed in 3% glutaraldehyde buffered with sodium cacodylate, embedded in epoxy and stained with toluidine blue. Images from light microscopy were captured and digitised (Cap view) and standardised (PhotoShop 7.0); then the selected sinusoidal areas of the pericentral vein areas were extracted with auto level function. Threshold in red images were obtained through Scion Image B4.02 analyser. Selected areas for quantification were measured as pixels per area (square inches). Standard referenced area utilized was 8.33 square inches^[25].

Table 1 Effect of different treatments on portal pressure (mean \pm SE)

Treatment	Portal pressure (PP) (mmHg)
Sham	7.9 \pm 0.6 ^b
PPVL	14.4 \pm 1.9
Sham + bilirubin	7.6 \pm 0.9 ^b
Sham + Sn-PPIX +	7.3 \pm 0.7 ^b
PPVL + Bilirubin	14.0 \pm 1.8
PPVL + Sn-PPIX	13.8 \pm 1.6

^b*P* < 0.001 between sham groups and PPVL, PPVL + Bilirubin and PPVL + Sn-PPIX groups, according to Neuman-Keuls' test.

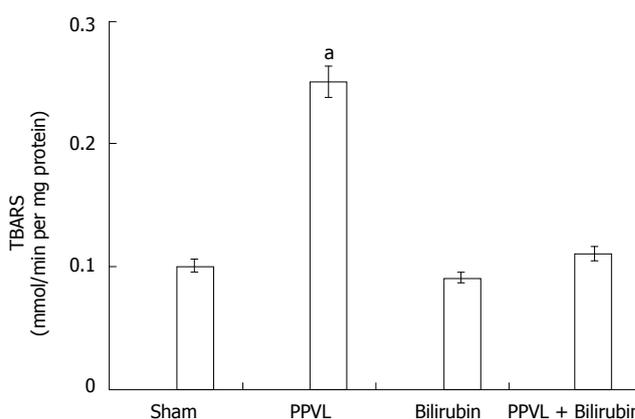


Figure 1 Effect of PPVL and bilirubin on lipid peroxidation. Rats were treated as described in Methods. Values are the means of three different experiments using three rats each time, and bars indicate SE. ^a*P* < 0.05 vs sham.

Protein determination

Protein concentration was measured following Lowry *et al.*^[26] using bovine serum albumin as standard.

Statistical analysis

Results are expressed as mean \pm SE. Data were analysed statistically by factorial analysis of variance (ANOVA) and followed by the Neuman-Keuls' test for comparison of means. Differences were considered significant at *P* < 0.05.

RESULTS

Portal pressure measurement

The portal pressure results are shown in Table 1. Differences between Sham *vs* PPVL rats at d 14 without and with bilirubin and Sn-PPIX were significant (*P* < 0.001). No differences were found either in Sham or in PPVL animals when they were treated with bilirubin and Sn-PPIX.

Oxidative stress generation

Reactive oxygen species are responsible for peroxidative cell damage. TBARS were 150% increased in PPVL rat livers (Figure 1).

GSH is a leading substrate for enzymatic antioxidant functions and is also a known radical scavenger. Therefore, if PPVL treatment induced the formation of ROS, it could be expected that GSH levels be affected. It can be

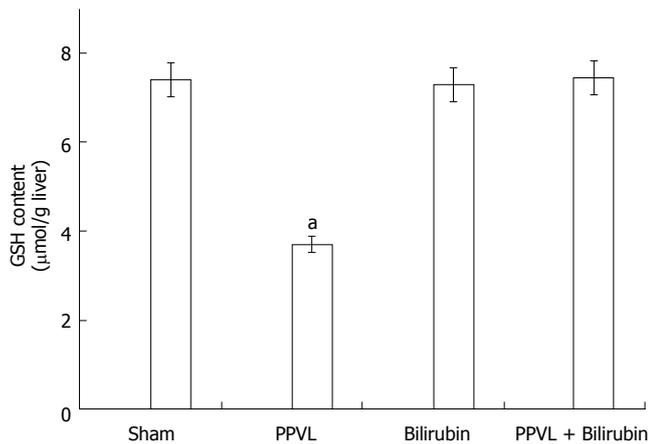


Figure 2 Effect of PPVL and bilirubin on GSH liver content. Rats were treated as described in Methods. Values are the means of three different experiments using three rats each time, and bars indicate SE. ^a*P* < 0.05 vs sham.

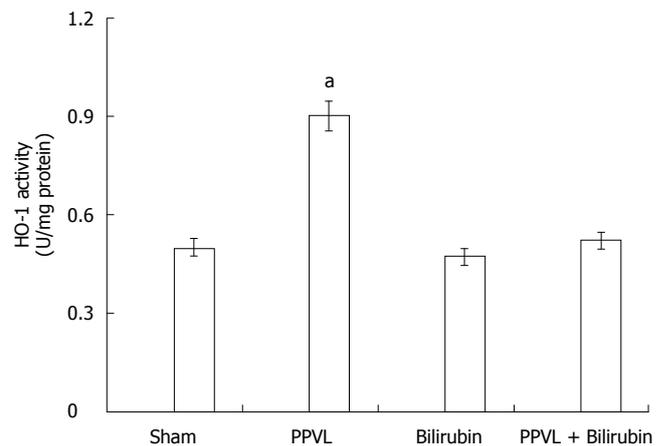


Figure 3 Effect of PPVL and bilirubin on HO-1 activity. Rats were treated as described in Methods. One unit of HO-1 is defined as the amount of enzyme producing 1 nmol of bilirubin per 30 min under the standard incubation conditions. Values are the means of three different experiments using three rats each time, and bars indicate SE. ^a*P* < 0.05 vs sham.

Table 2 Effect of PPVL and bilirubin on antioxidant enzyme activities (mean ± SE)

Group	Catalase (pmol/mg protein)	Total superoxide dismutase (U/mg protein) ¹	Glutathione peroxidase (U/mg protein) ²
Sham	10.5 ± 1.1 ^a	9.0 ± 0.2 ^b	0.129 ± 0.004 ^a
PPVL	6.5 ± 0.5	4.5 ± 0.1	0.100 ± 0.004
Sham + Bilirubin	10.7 ± 0.9 ^a	9.3 ± 0.1 ^b	0.132 ± 0.006 ^a
PPVL + Bilirubin	9.4 ± 0.9 ^a	8.9 ± 0.2 ^b	0.131 ± 0.004 ^a

Enzymatic activities were assayed as described in the text. ¹One unit of SOD activity is defined as the amount of enzyme required to inhibit 50% of the epinephrine autooxidation. ²One unit of the enzyme represents the decrease of 1 mmol of NADPH/min under the assay conditions. ^a*P* < 0.05 between Catalase and Glutathione Peroxidase of Sham groups, PPVL + Bilirubin and PPVL group, ^b*P* < 0.01 between Superoxide Dismutase of group of Sham groups, PPVL + Bilirubin and PPVL group and PPVL group according to Neuman-Keuls' test.

seen in Figure 2 that in the liver of PPVL animals GSH concentration was about 50% decreased when compared to Sham group. When rats were pretreated with bilirubin both TBARS and GSH levels were equal to Sham animals (Figures 1 and 2).

Enzymatic defence system

Table 2 shows the activities of CAT, SOD and GSH-Px in the liver of Sham and PPVL animals pretreated with bilirubin as well as in both groups without bilirubin. These enzymes were significantly inhibited (CAT 38%, SOD 50% and GSH-Px 23%) in PPVL rats. Pretreatment with bilirubin had no effect in Sham animals, but in PPVL rats the inhibition of antioxidant enzymes was totally prevented.

Heme oxygenase activity and expression

As it is shown in Figure 3, PPVL treatment increased liver HO-1 activity by 80%. Bilirubin pretreatment did not have any effect in Sham rats, but it completely prevented HO-1 induction provoked by PPVL. As occurred with HO-1 activity, a similar increment in HO-1 expression was obtained in liver of PPVL rats (Figure 4A and B).

Previous results using acute intoxication with different drugs demonstrated that as a consequence of ROS pro-

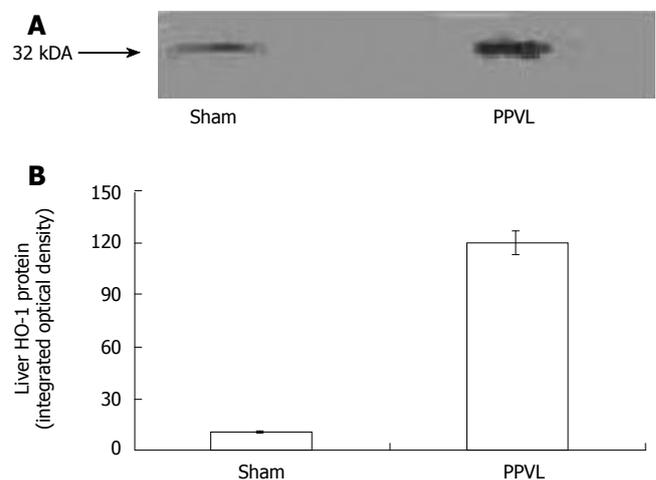


Figure 4 Western blot analysis of HO-1 expression in PPVL rat liver (A). Densitometry was done to quantify HO-1 protein expression (B). The blot is representative of 3 blots with a total of 4-5 samples/group between the 3 blots. *P* < 0.01 sham vs PPVL group.

duction, liver GSH levels rapidly decreased and then HO-1 was induced. It was also observed that HO-1 induction appeared to occur once the ROS was increased and the antioxidant defence system decreased. Bilirubin action as a protective agent against oxidative stress suggests that inhibition of HO-1 could enhance even more the oxidative stress induced by PPVL model. Therefore, the effect of Sn-PPIX, a strong inhibitor of HO-1 was assayed 24 h before the end of the experiment. Table 3 shows that a single administration of Sn-PPIX decreased to half HO-1 activity in Sham rats. PPVL animals pretreated with the inhibitor of HO-1 (Sn-PPIX), slightly decreased hepatic GSH levels (20%) and significantly increased TBARS production (70%) over the values obtained by PPVL treatment.

Effect of CO on hepatic sinusoidal vasodilatation

As it is shown in Table 4 and Figure 5, CO produced a significant increase in the sinusoidal hepatic areas of PPVL rats when compared to sham animals. Pretreatment of

Table 3 Effect of Sn-PPIX administration on the HO-1 induction and on TBARS and GSH contents in PPVL rat livers (mean \pm SE)

Treatment	HO-1 (U/mg protein) ¹	TBARS (nmol/min per mg protein)	GSH content (μ mol/g liver)
Sham	0.50 \pm 0.01 ^b	0.10 \pm 0.01 ^d	7.5 \pm 0.2 ^d
PPVL	0.90 \pm 0.02	0.25 \pm 0.01	3.7 \pm 0.1
Sham + Sn-PPIX	0.25 \pm 0.01	0.11 \pm 0.01 ^d	7.2 \pm 0.3 ^d
PPVL + Sn-PPIX	0.48 \pm 0.04	0.32 \pm 0.02	2.3 \pm 0.2

Sn-PPIX was administered as described in Materials and Methods. Sham animals were injected with saline solution. Enzymatic activity was assayed as described in the text. ¹One unit of the enzyme forms 1 nmol of bilirubin/30 min under assay conditions. Different letters within columns indicate significant differences according to Neuman-Keuls' test. Differences in HO-1 were of ^b*P* < 0.01 between Sham group and PPVL, Sham + Sn-PPIX groups. Differences in TBARS and in GSH content were of ^d*P* < 0.01 between Sham groups and PPVL groups.

Table 4 Effect of different treatments on vasodilatation of hepatic sinusoids (mean \pm SE)

Treatment	Sinusoidal area
Sham (<i>n</i> = 258)	3.64 \pm 0.08
PPVL (<i>n</i> = 245)	4.20 \pm 0.02 ^b
Sham + Sn-PPIX (<i>n</i> = 257)	3.45 \pm 0.01
PPVL + Sn-PPIX (<i>n</i> = 249)	3.48 \pm 0.04

The selected area for quantification was measured as pixels per area (square inch). The area standard used was 8.33 square inch. ^b*P* < 0.01 between PPVL and the other three groups, according to Neuman-Keuls' test.

PPVL animals with Sn-PPIX totally prevented this effect and similar values to Sham group were obtained (Table 4). Groups Sham and PPVL + Sn-PPIX showed normal histological features when focusing on sinusoidal space (similar Figure 5A), while group PPVL showed sinusoidal dilation (Figure 5B).

DISCUSSION

Aerobic organisms are continuously exposed to oxygen, which renders them prone to damage generated by ROS. There are a number of cellular mechanisms to protect the cell from oxidative stress. Antioxidant enzymes such as CAT, SOD and GSH-Px play critical roles in oxidative stress protection by converting ROS into less harmful products^[27]. Recently, HO-1 and its product bilirubin have gained attention in cytoprotection against oxidant mediated injury^[10-14,28,29].

Our results showed that the administration of bilirubin in rats decreased PPVL-induced lipid peroxidation, restored GSH content and activity of the antioxidant enzymatic system to normal levels. These results open possibilities for considering the use of bilirubin as an efficient antioxidant and free radical scavenger (Figures 1 and 2, Table 2).

Bilirubin administration totally prevented the decrease of antioxidant enzyme activities and induction of HO-1 activity provoked by PPVL (Table 2, Figure 3). These findings are in agreement with our own previous reports

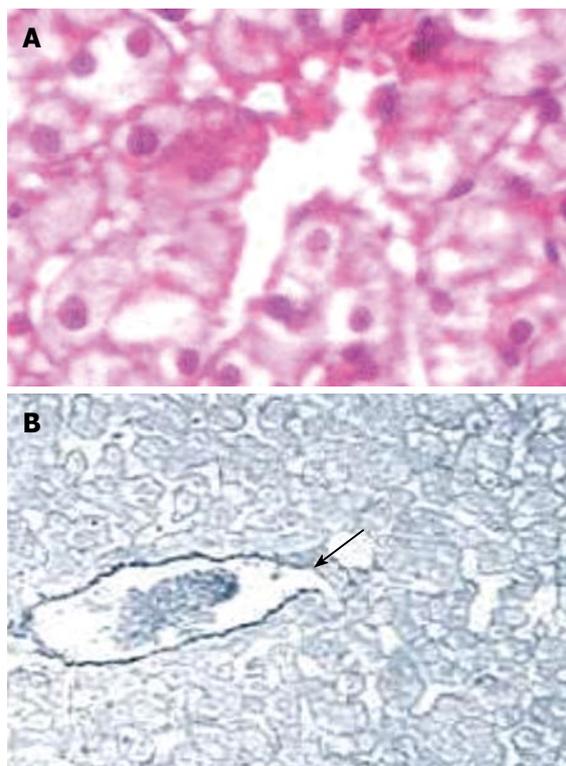


Figure 5 Hepatic centrolobular zone of Sham operated rats (A). Sinusoidal area spread normal histological features (HE, 100 x magnification). Hepatic centrolobular zone corresponding to PPVL group (B). Sinusoidal area is increased (arrows) (Reticulin, enhanced image, 40 x magnification).

on the protective effect of bilirubin against oxidative stress caused by different physical and chemical agents^[28].

Administration of Sn-PPIX, a known competitive inhibitor of HO-1 expectedly decreased HO-1 activity (Table 3). When the inhibitor was given to PPVL rats, HO-1 activity remained at control levels, but an increase in TBARS content and a decrease in GSH levels were observed (Table 3).

CO is, like nitric oxide, an endogenous compound that activates guanylate cyclase^[30], leading to the generation of cyclic guanosine monophosphate, which in turn mediates various physiological functions, and excessive production of CO, a consequence of HO-1 overexpression, could play an important role in modulating vascular tone under different pathological situations^[16,31]. Besides, it has been demonstrated that administration of zinc protoporphyrin IX, a strong inhibitor of heme oxygenase, elicits a marked increase in the vascular resistance as a consequence of sinusoidal constriction^[32]. These results are in agreement with the effects observed in PPVL rats pretreated with Sn-PPIX (Table 4, Figure 5). Increased production of CO, as a consequence of HO-1 induction, may be beneficial in some pathological situations, including hypertension^[33], but can also be detrimental in other disease conditions^[34]. For instance, in PH it would be detrimental because an increased production of CO in splanchnic organs may contribute to the development and maintenance of the splanchnic hyperdynamic circulation associated with the PH syndrome. But at this point, we can also speculate that an enhanced release of the vasodilator CO may contribute to the modulation of hepatic blood flow.

It has been demonstrated that HO-1 is induced in

splanchnic organs of PH rats^[35-37], and it was also reported that patients with portal hypertensive diseases had significantly greater the activity and expression of liver HO-1 than those from normal individuals^[38]. HO-1 activity in the liver is not related to systemic vascular resistance; however this organ may show high activity of HO due to that reticuloendothelial cell-rich tissues are involved in the removal of senescent erythrocytes and plasmatic hemoglobin from the circulation.

The precise mechanisms whereby HO-1 expression is induced in PH rats are still unknown, but the results here obtained lead us to believe that this induction was provoked as a consequence of oxidative stress generation. Besides, it is noteworthy that HO-1 is transcriptionally activated by several chemical and physical factors that may be increased during PH^[1], including cytokines, endotoxin and shear stress^[39]. For these reasons, in the present study we suggest a beneficial role of HO-1 overexpression in a rat model of prehepatic portal hypertension.

In this study, no differences in portal pressure were observed in PPVL rats when compared this group with PPVL + Bilirubin and PPVL + Sn-PPIX groups. This fact could be explained by the persistence of some amount of portal systemic shunting and by the hepatic blood resistance that was not modified despite the pericentral sinusoidal dilation documented here.

In conclusion, our data suggest that ROS generation by portal hypertension situation alter heme degradation. They also evidenced that the cell possesses an inducible pathway against oxidative stress finally leading to increased bilirubin formation, which then exerts its antioxidant protective action. These results shed light on the response of heme degradation to PPVL-induced oxidative stress, and further support the involvement of bilirubin as a physiological protective agent against PPVL-induced oxidative cell injury. The potential use of bilirubin as an antioxidant in combination with other antioxidants or with conventional treatments, as a new therapeutic approach for portal hypertensive patients, can be considered.

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BASIC RESEARCH

Changes of inducible protein-10 and regulated upon activation, normal T cell expressed and secreted protein in acute rejection of pancreas transplantation in rats

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Abstract

AIM: To investigate the role of IFN- γ inducible protein-10 (IP-10) and regulated upon activation, normal T cell expressed and secreted (RANTES) protein in acute pancreatic allograft rejection in rats.

METHODS: An experimental pancreas transplantation model was established using diabetic SD rats as the recipient, induced by applying streptozocin (STZ). Pancreas transplantation was performed with a physiologic method of portal venous and enteric drainage. Rats were divided into two groups, isograft group (group A, $n = 24$) and allograft group (group B, $n = 24$) in which either healthy SD rats or Wistar rats served as donors, respectively. Twelve diabetic or healthy SD rats were used as controls. At d 1, 4, 7, and 10 post transplantation, serum IP-10 and RANTES were assessed by ELISA and their expression in the allografts was determined by immunohistochemistry.

RESULTS: In group B (allograft group), the development of acute rejection was significantly correlated with increased serum concentration and tissue expression of IP-10 and RANTES, with a peak level at d 7 post transplantation. In contrast, there was no obvious change before and after transplantation in group A (isograft group).

CONCLUSION: Our study suggests a possible role of IP-10 and RANTES in acute rejection and early monitoring of chemokines may be helpful in predicting the outcome of pancreas transplantation.

INTRODUCTION

In the past several decades, great success has been made in the transplantation field. However, the acute rejection is still a tremendous obstacle to the development of the transplantation. Acute allograft rejection is a complex process comprising interrelated series of events, such as the recognition of the allograft antigen, the activation and proliferation of the leukocytes, the migration of the leukocytes to the allograft. In the pathogenic process, several kinds of inflammatory molecules are required, such as proinflammatory cytokines, adherence molecules and chemokines. The chemokines are a large family of "chemoattractant cytokines", including IFN- γ inducible protein-10 (IP-10), regulated upon activation, normal T cell expressed and secreted (RANTES), Mig, *etc.* They play a critical role in directing leukocytes to the allograft and in amplifying intragraft inflammation during rejection^[1,2]. In the past years it has become evident that individual proinflammatory chemokines (such as IP-10, RANTES) are indispensable in the rejection process of heart transplantation, kidney transplantation, and lung transplantation, compared to other chemokines^[3-5]. However the roles of chemokines in pancreas transplantation are still not well known and pancreas transplantation has its own speciality that differs from other organ transplantations. For example, it is well known that pancreatic grafts are very susceptible to allograft rejection because of the strong immunogenicity of pancreas itself^[6]. To assess the changes of chemokines IP-10 and RANTES in the acute rejection of pancreas transplantation, we established the pancreas transplantation model in rats firstly, using a physiologic method of portal

venous and enteric drainage^[7], and then evaluated the severity of rejection, detected the concentration of serum RANTES and IP-10 using ELISA kits, and examined the expressive position and intensity of IP-10 and RANTES in the allograft pancreas.

MATERIALS AND METHODS

Animal preparation

The diabetic rats were induced firstly by single intravenous injection of streptozocin (STZ; Sigma, USA) at a dose of 50 mg/kg of body weight, when the closed flock male SD rats weighing 250-280 g were chosen (offered by the Experimental Animal Center of Jiangsu Province, China). After the STZ injection, the nonfasting blood glucose and urine glucose of the rats were assayed before the inducement and measured on alternate day. Only rats with nonfasting blood glucose exceeding 16.8 mmol/L and the strong positive reaction of the urine glucose were selected as recipients. The closed flock male Wistar rats (offered by the Silaike Co. Ltd, Shanghai, China) and healthy SD rats weighing 220-250 g served as donors. The donors and recipients were matched under the condition that the weights of the recipients were more than that of donors by about 30 g.

Grouping of experimental rats

Rats were divided into two groups, isograft group (group A, $n = 24$) and allograft group (group B, $n = 24$), in which either healthy SD rats or Wistar rats served as donors, respectively. Twelve diabetic or healthy SD rats were used as controls.

Surgical procedure and collection of specimen

A physiologic method for pancreas transplantation was adopted, in which the vein was reconstructed by end-to-side anastomosis between the donor portal vein and the recipient superior mesenteric vein, and arterial reconstruction was carried out by end-to-side anastomosis of the donor to the recipient abdominal aorta, and enteric drainage was performed by a side-to-side anastomosis between the duodenum of donors and that of recipients. The level of the recipient's blood glucose below 11.2 mmol/L at 1 d post operation was regarded as successful transplantation. The recipients were sacrificed at 1, 4, 7, 10 d ($n = 6$ animals/time point) after transplantation. The 12 rats in control groups were killed at the beginning of the experiment. Blood samples were collected and placed quietly for clotting for 2 h at room temperature before centrifuging for 30 min at $1000 \times g$, then the serum was pipetted immediately and stored at -70°C . After representative portions of pancreas grafts were removed, some of them were immediately snap-frozen in liquid nitrogen for immunohistology and the rest were fixed in 10% formalin for histopathological examination.

Histopathology examination

The samples of pancreas grafts were fixed, dehydrated, embedded, sliced, and stained with hematoxylin and eosin following the routine proposal. The classification of acute rejection was stated according to the Nakhleh

Table 1 Classification of acute rejection in allograft group post transplantation

	Grade I	Grade II	Grade III	Grade IV
1 d	5	1		
4 d		4	2	
7 d			5	1
10 d				6

Nakhleh Classification Criterion, Grade I: no rejection present, Grade II: mild lymphoplasmacellular infiltration or endothelialitis, Grade III: moderate lymphoplasmacellular infiltration or arteritis/vasculitis, Grade IV: severe lymphoplasmacellular infiltration or fibrinoid necrosis.

Classification Criterion^[8].

Determination of serum IP-10 and RANTES

ELISA kits (TPI INC., USA) were used for the determination of serum IP-10 and RANTES, and the procedure was strictly according to the protocol recommended by the manufacturers. The results were expressed as the quantity per mL serum.

Immunohistology

For immunohistology, 10 μm frozen sections of pancreas were prepared, fixed in acetone for 10 min, dried in the airy place, and incubated with goat polyclonal IP-10 antibodies and rabbit polyclonal RANTES antibodies respectively. Then, the sections were incubated with rabbit anti-goat IgG and goat anti-rabbit IgG respectively. All the reagents were offered by Santa Cruz Co, USA. The cells stained clearly were regarded as positive ones. According to the percentage of positive cells in the whole infiltrating immune cells, the results of immunohistology were expressed in four grades: negative (the rate of positive cells $< 5\%$), mild positive (the rate of positive cells $> 5\%$ and $< 25\%$), moderate positive (the rate of positive cells $> 25\%$ and $< 50\%$), strong positive (the rate of positive cells $> 50\%$).

Statistical analysis

The concentration of serum IP-10 and RANTES were expressed as mean \pm SD. The significance of differences was tested using either *t*-test for means of two samples or analysis of Variance and *q*-test for means of multiple samples. $P < 0.05$ was considered as significant.

RESULTS

Classification of acute rejection

The acute rejection was classified according to the criterion stated by Nakhleh. In this study, a mild edema appeared around the islet and the acinus 1 d post transplantation both in the allograft and isograft groups. The edema disappeared and no evident rejection was found at 4, 7, and 10 d after the operations in the isograft group, though evident rejection appeared in the allograft group (Table 1).

Changes of serum IP-10 and RANTES

In the allograft group, serum IP-10 concentration was elevated significantly since 4 d and peaked at 7 d after the

Table 2 Concentration of serum IP-10 in isograft, allograft and control groups (ng/L)

	1 d	4 d	7 d	10 d
Isograft group	40.88 ± 10.76	36.74 ± 10.33	38.13 ± 12.73	31.83 ± 7.55
Allograft group	43.34 ± 15.29	66.26 ± 11.08 ^a	83.28 ± 16.44 ^a	70.08 ± 17.65 ^a
Control group	28.76 ± 7.41			

^a $P < 0.05$ vs control group.

Table 3 Concentration of serum RANTES in isograft, allograft and control groups (ng/L)

Group	1 d	4 d	7 d	10 d
Isograft	357.87 ± 63.26 ^a	274.77 ± 58.22	265.87 ± 43.40	267.55 ± 48.91
Allograft	388.48 ± 104.45 ^a	586.72 ± 90.06 ^a	746.28 ± 92.64 ^a	631.49 ± 106.34 ^a
Control	251.18 ± 44.94			

^a $P < 0.05$ vs control group.

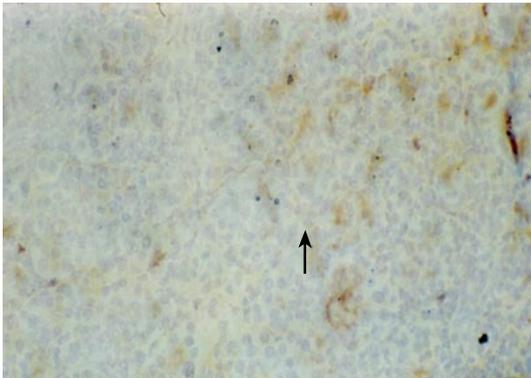


Figure 1 IP-10 immunohistochemical staining of pancreas allografts. Mild expression was observed at 1 d post transplantation ($\times 200$).

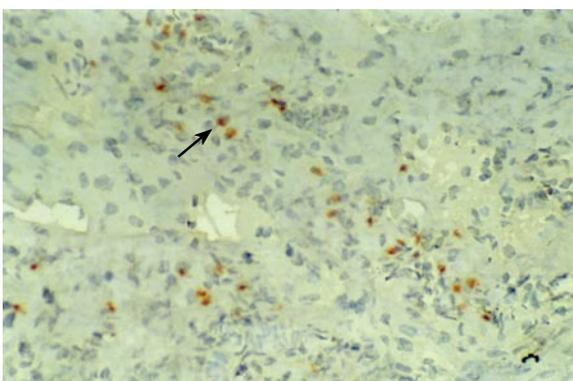


Figure 2 RANTES immunohistochemical staining of pancreas allografts. Mild expression was observed at 1 d post transplantation ($\times 200$).

operations, compared to the control group ($P < 0.05$). However, no significant difference was found between the isograft group and the control group at the four corresponding phases. The tendency of serum RANTES was similar to that of IP-10, only showing a sharp increase at 1 d after the transplantation in the isograft group ($P <$

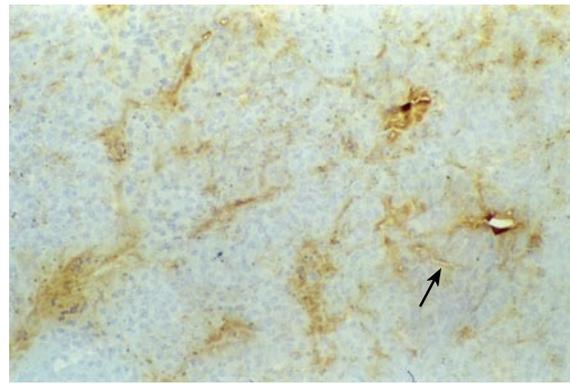


Figure 3 IP-10 immunohistochemical staining of pancreas allografts. Moderate expression was observed at 4 d post transplantation ($\times 200$).

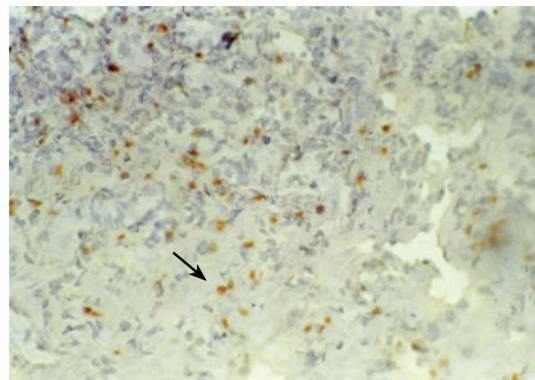


Figure 4 RANTES immunohistochemical staining of pancreas allografts. Moderate expression was observed at 4 d post transplantation ($\times 200$).

0.05) compared with the control group (Tables 2 and 3).

Expression of IP-10 and RANTES in the pancreas grafts

There were no detectable expressions of IP-10 and RANTES protein in the normal pancreas. Mild expression was observed at 1 d after the operation both in the allografts and isografts (Figures 1 and 2). At 4 d after the transplantation, expression of IP-10 and RANTES appeared moderately in the allografts (Figures 3 and 4), whereas their expression could not be detected in the isografts. At 7 d after the operation, the positive expression was strongest in the allografts (Figures 5 and 6). Besides, they were expressed in different places. IP-10 was gathered around the vessels; RANTES was clearly expressed in the lymphocytes in interstitial tissues and concentrated in the narrow space between the inflammatory and normal tissues.

DISCUSSION

Acute rejection is a very complicated process, in which the cellular and humoral immune mechanisms are involved. In the process of acute rejection, the cellular immune reaction is of primary importance^[9]. The circulating lymphocytes firstly adhere to the vessel endothelium, then penetrate the vessel wall, cross the interstitial tissue, migrate into the graft, and finally destroy the graft^[10]. In these processes, the chemokines are indispensable.

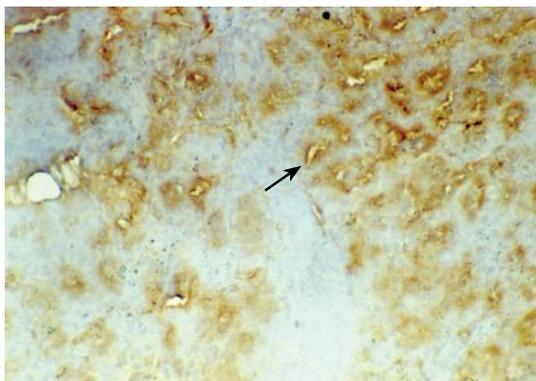


Figure 5 IP-10 immunohistochemical staining of pancreas allografts. Strong positive expression was observed at 7 d post transplantation ($\times 200$).

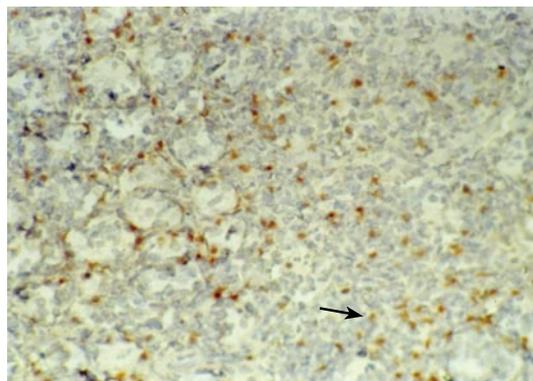


Figure 6 RANTES immunohistochemical staining of pancreas allografts. Strong positive expression was observed at 7 d post transplantation ($\times 200$).

Chemokines accelerate the migration and activation of the leucocytes and lymphocytes through the interaction between the chemokines and their receptors, and play a key role in recruitment of inflammatory cells into an organ transplant^[11].

Chemokines are a group of 8-11 kDa proteins, owning four conservative tyrosines in their primary sequence. According to the positional relation between the first tyrosine and the second one, the family are divided into four subfamilies, namely, CXC subfamily, CC subfamily, C subfamily and CX₃C subfamily^[12].

IP-10 is one of CXC subfamily, mainly coming from activated fibroblasts, monocytes, endothelial cells and T cells^[13]. By the interaction between IP-10 and its receptor (CXCR3), IP-10 function in the recruitment of activated Th1 cells specifically and amplify the Th1 reaction. It has been reported that IP-10 and CXCR3 were detected in the allograft in the lung and heart transplantation, and the quantities of IP-10 and CXCR3 are positively related with the severity of rejection response^[14]. The latest study performed by Kanmaz *et al*^[15] showed an important correlation between urinary excretion of IP-10 and acute rejection in baboon kidney transplantation and indicated that urinary IP-10 might be a more accurate predictive parameter than serum creatinine to monitor the occurrence of acute rejection of renal transplant.

RANTES belongs to the CC subfamily, produced and secreted by the CD8⁺ T cells, endothelial cells, and fibroblasts, directing the recruitment of T cells, monocytes, eosinophils to the local tissue^[16]. It has been confirmed that RANTES is closely related to the pathogenesis of rejection in heart, lung, kidney transplantation, *etc.* Mulligan *et al*^[2] found that the protein of RANTES was detectable 6-8 d after the heart transplantation in mice in heterogeneous group. However, the results were opposite in the homogeneous heart transplantation. Sekine *et al*^[17] reported that the mRNA of RANTES was closely positively correlated with the number of infiltrating monocytes, using the models of lung transplantation. Similar results were also obtained in liver transplantation^[18]. Some experiments have succeeded in prolonging the functional time of the allografts by inhibiting the interaction between the chemokines and their receptors. Hancock *et al*^[19] reported that the survival phase was elon-

gated, provided that the IP-10 knockout mice served as the donors. Yun *et al*^[20] demonstrated that the use of Met-RANTES, the specific antagonist to RANTES, could alleviate the infiltration of CD4⁺ and CD8⁺ lymphocytes and attenuate allograft rejection in mice. It seems that IP-10 and RANTES play a pivotal role in the rejection of organ transplantation.

In our experiments, there was no detection of IP-10 and RANTES protein in the normal pancreas. In the isograft group, the proteins of IP-10 and RANTES were detected weakly at 1 d, and restored to the normal level since 4 d post transplantation. In the allograft group, the expression of IP-10 and RANTES protein were also upregulated in the primary phase of acute rejection, and then developed with the severity of the rejection reaction. The trend of serum IP-10 and RANTES was the same as that in the graft. It seems that the detection of IP-10 and RANTES benefits the early diagnosis of acute rejection.

We also noticed that the expressing loci and pattern of IP-10 and RANTES was obviously different. IP-10 was gathered around the vessels; meanwhile, RANTES was clearly expressed in the lymphocytes in the interstitial tissues and crowded in the narrow space between the inflammatory and normal tissues. This result perhaps demonstrated that IP-10 and RANTES play different roles in the acute rejection. IP-10 is a key molecule in directing the lymphocytes to penetrate the endothelium and migrate across the tissue. RANTES, produced by the cells in the inflammatory tissues, can be recognized and bound with its receptors on the surface of passage macrophage, T cells. The activated T cells unregulated the expression of RANTES, and more inflammatory cells were attracted into the graft. The process was cycled and amplified and the inflammation aggravated.

In summary, IP-10 and RANTES play a critical role in the acute rejection after pancreas transplantation. Further research will help the understanding of the pathogenesis of acute rejection. IP-10 and RANTES will be a tool for the early diagnosis and a target for the therapy of acute rejection.

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Clinicopathological and immunohistochemical analysis of gastrointestinal stromal tumor

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of p21WAF1 and Bax plays an important role in potential malignancy and malignancy rather than in benign GIST. p21WAF1 and Bax may be used as the markers in the assessment of GIST malignant potential.

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Key words: Gastrointestinal stromal tumor; p21WAF1; Bax

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Abstract

AIM: To investigate the clinicopathological features of gastrointestinal stromal tumor (GIST) and to study the reference indexes for malignancy.

METHODS: Fifty-two cases of primary GIST were distinguished from a group of gastrointestinal mesenchymal tumors using a panel of antibodies such as CD117 and CD34 by immunohistochemical SP method. Their biological behaviors were analyzed using the expression of p21WAF1 and Bax in 52 cases of GIST.

RESULTS: Grossly, the tumor size was between 1.5 cm and 13 cm (mean: 5.5 cm). Focal areas of hemorrhage, necrosis, or small cyst formation could be seen. Microscopically, the tumor was composed of spindle cells (20 cases), epithelioid cells (20 cases) and mixed cells (12 cases). Immunohistochemically, CD117 and CD34 showed diffuse strong positive expressions, the positive rates were 98.1% and 92.3%. SMA, S-100, NSE, NF and MBP showed focal positive expressions, the positive rates were 48.1%, 28.8%, 25%, 21.2% and 42.3% respectively. Vimentins were all positive desmin and CgA were all negative. In normal adult stomach and intestine, the immunoreactive staining for CD117 and CD34 showed immunoreactive interstitial cells of Cajal in myenteric neuroplexus. Among the 52 cases of GIST, 27 were positive for p21WAF1 (51.9%), 29 for Bax (55.8%). The expression of p21WAF1 and Bax had no significant difference with the localization, size, histological subtype of GIST, but had a significant difference with the histological grade ($P = 0.000$, respectively). p21WAF1 expression had a positive correlation to Bax expression ($r = 0.461$, $P = 0.001$, $\kappa = 0.459$).

CONCLUSION: GIST has complicated arrangements and various cell types. Positivity of CD117 and CD34 is the most valuable factor in diagnosing GIST. Expression

INTRODUCTION

Gastrointestinal stromal tumor (GIST) is the most common mesenchymal tumor in the digestive tract^[1]. Mutational activation of *c-kit* has been found to be associated with the pathogenesis of GIST^[2], CD117 positivity is the most valuable marker in diagnosing GIST. But the biological behaviors of GIST are difficult to predict^[3], some metastasize whereas others remain asymptomatic for years^[4]. In this study, we detected CD117, CD34, SMA, S-100, NSE, NF, MBP, vimentin, desmin, CgA, p21WAF1 and Bax using immunohistochemical staining method to explore the expression of p21WAF1 and Bax and its correlation to clinicopathologic characteristics of GIST.

MATERIALS AND METHODS

Specimens

Fifty-two cases of GIST were selected from the Department of Pathology, First Hospital of Harbin Medical University in 2001 to 2004. The slides stained with hematoxylin and eosin were reviewed. Based on the diagnostic criteria proposed by Haber *et al*^[5], among the 52 cases of GIST, 20 were cases of benign GIST, 12 and 20 were cases of potentially malignant and malignant GIST respectively. Age ranged 34-78 years (mean: 54.3 years).

Immunohistochemistry

Resected specimens were fixed in 40 g/L formaldehyde and embedded in paraffin. Four- μ m thick sections were dewaxed, rehydrated in graded alcohols, and processed

Table 1 Primary antibody used for immunohistochemical study

Antibody	Clone	Dilution	Pretreatment
CD117	polyclonal	instant	Microwave
CD34	QBEnd/10	instant	Microwave
SMA	1A ₄	instant	None
Vimentin	V9	instant	None
Desmin	ZC18	instant	None
S-100	4C4.9	instant	None
NSE	E27	instant	None
NF	DA2/FNP7	instant	Microwave
MBP	polyclonal	instant	None
CgA	LK2H10	instant	Microwave
p21WAF1	4D10	1:25	Microwave
Bax	2D2	1:50	Microwave

using immunohistochemical SP method. All antibodies were purchased from Beijing Zhongshan Biotechnology CO. LTD (Table 1). Tissues positive for all the purchased antibodies were used as positive controls, sections prepared with PBS instead of the primary antibody were used as negative controls. When the number of positive cells was < 10%, 10%-50%, or > 50%, the immunoreactivity for p21WAF1 and Bax was scored as 1 +, 2 +, 3 +, respectively. When the number of positive cells was ≤ 50% and > 50%, the immunoreactivity for other antibodies was scored as 1 +, 2 +, and 3 +, respectively.

Statistical analysis

Statistical analyses were performed using SPSS 11.5 software. Pearson χ^2 test, Fisher's exact test, Spearman rank correlation test and Kappa test were used when appropriate. $P < 0.05$ was considered statistically significant.

RESULTS

Grossing findings

The tumor size was 1.5-13 cm (mean: 5.5 cm). Tumors were generally round or oval in shape with pink-white and firm well-circumscribed and cut surface. Focal areas of hemorrhage, necrosis, or small cyst formation could be seen. Submucosal or subserosal tumors sometimes extended into the gastrointestinal lumen, leading to ulceration in mucosa.

Light microscopic findings

Among the 52 cases of GIST, spindle cell type was found in 20, epithelioid cell type in 20 (Figure 1A) and mixed type in 12. The tumor cells arranged in interlacing fascicles or formed whirls. The tumor cells were spindle, oval or round in shape, sometimes signet-ring like cells could be observed with a clear cytoplasm (Figure 1B). Hemorrhage and/or necrosis and/or hyaline degeneration (Figure 1C) could be found in some cases. Mucosal or serosal invasion (Figure 1D) sometimes could be seen in some malignant GISTs.

Table 2 Relation between p21WAF1, Bax expression and clinicopathological features

Items	<i>n</i>	p21WAF1		Bax		<i>P</i>		
		positive %	<i>P</i>	positive %	<i>P</i>			
Localization	Stomach/ Intestine	36	17	47.2	0.309	18	50	0.209
		Others	16	10	62.5		11	68.8
Tumor size	< 5 cm	21	12	57.1	0.535	13	61.9	0.463
	≥ 5 cm	31	15	48.4		16	51.6	
Histological subtype	Spindle	20	11	55	0.720	9	45	0.086
	Epithelioid	20	9	45		10	50	
	Mixed	12	7	58.3		10	83.3	
Histological grade	Benign	20	1	5	0.000	2	10	0.000
	Potentially malignant	12	9	75 ^b		11	91.7 ^b	
	Malignant	20	17	85		16	80	

^b $P < 0.01$.

Table 3 Correlation between expressions of p21WAF1 and Bax

p21WAF1	<i>n</i>	Bax		<i>r</i>	<i>P</i>
		+	-		
+	27	21	6	0.461	0.001
-	25	8	17		

Immunohistochemical findings

CD117 and CD34 showed diffuse positive expressions, the positive rates were 98.1% (Figure 2A) and 92.3%. SMA, S-100, NSE, NF and MBP showed focal positive expressions, the positive rates were 48.1%, 28.8%, 25%, 21.2% and 42.3%, respectively. Vimentins were all positive while desmin and CgA were all negative. In normal adult stomach and intestine, the immunoreactive staining for CD117 and CD34 showed immunoreactive interstitial cells of Cajal in myenteric neuroplexus (Figure 2B). Among the 52 cases of GIST, 27 were positive for p21WAF1 (51.9%, Figure 2C), 29 for Bax (55.8%, Figure 2D).

According to the χ^2 test, the expression of p21WAF1 and Bax had no significant differences in the localization, size, and histological subtype of GIST, but there was a significant difference in the histological grade ($P = 0.000$, Table 2). There was a significant difference between benign and potentially malignant or malignant GISTs, but the difference in p21WAF1 and Bax expression between potentially malignant and malignant GISTs was not significant. According to Spearman rank correlation test and Kappa test, p21WAF1 expression had a positive correlation to Bax expression ($r = 0.461$, $P = 0.001$, $\kappa = 0.459$, Table 3).

DISCUSSION

In 1983, Mazur and Clark^[6] first introduced the vague term 'gastrointestinal stromal tumor'. Under light microscope,

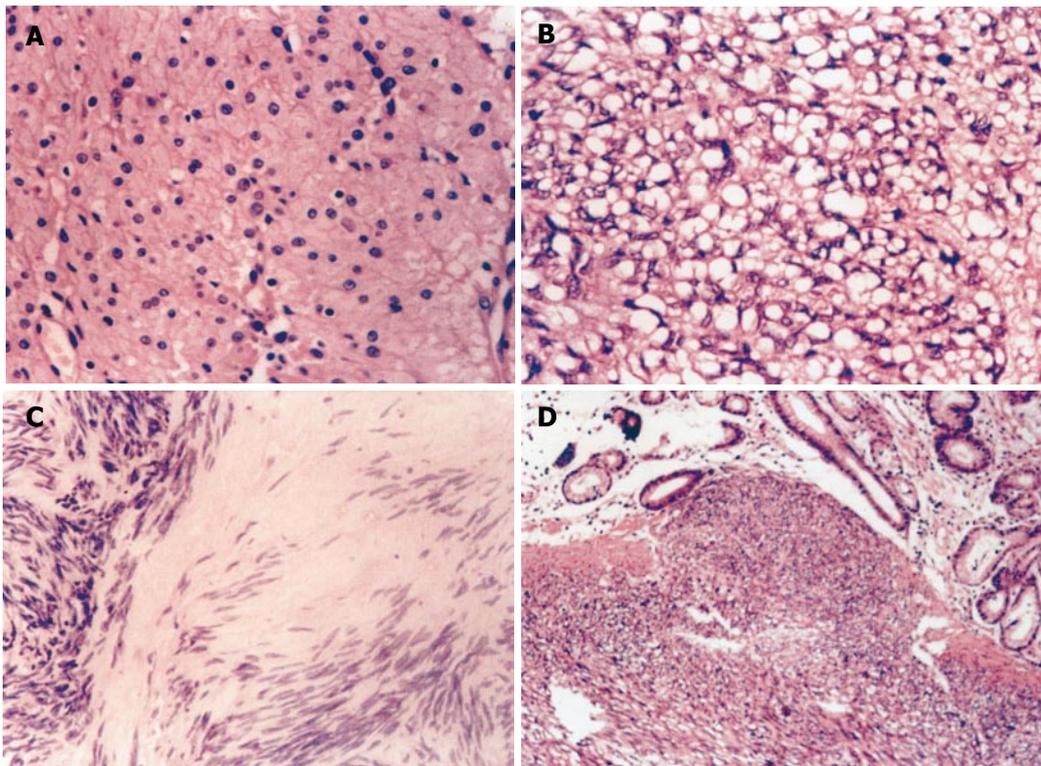


Figure 1 Epithelioid cells (A), signet-ring like cells (B), hyaline degeneration (C), and mucosal invasion (D) in GISTs.

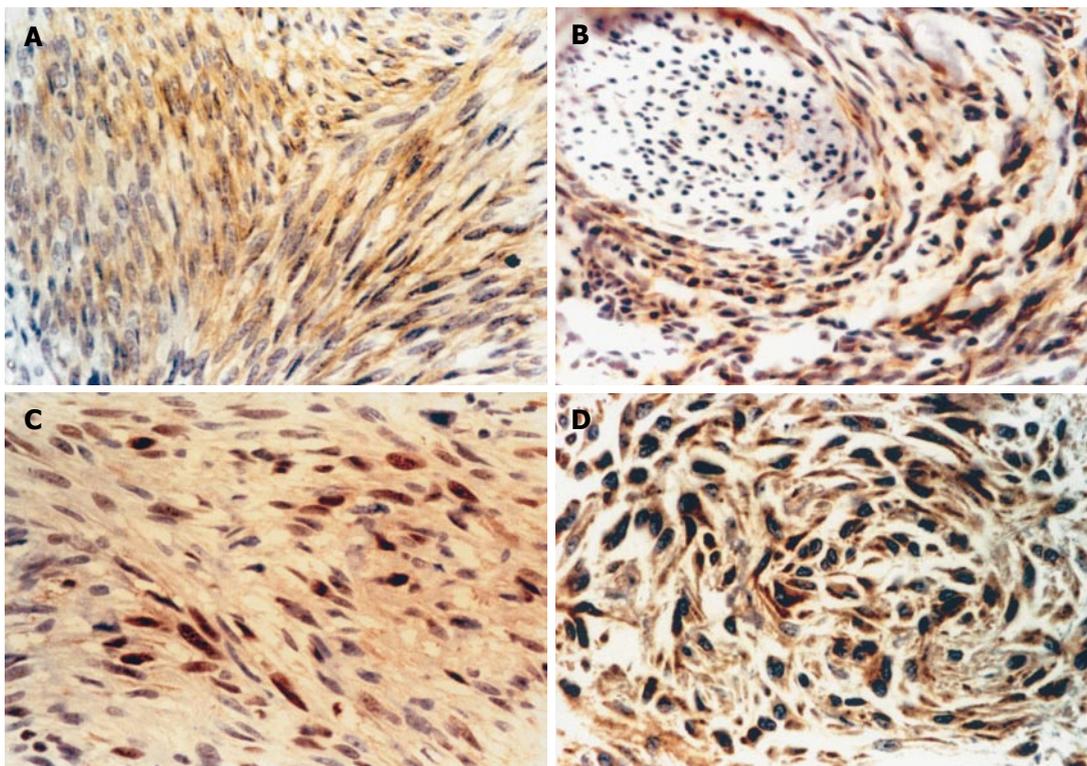


Figure 2 Expressions of CD117 (A), CD34 (B), p21WAF1 (C), and Bax (D) in GIST.

the morphology of stromal tumors looks sometimes like a leiomyoma, sometimes like a Schwannoma^[7]. Most gastrointestinal mesenchymal tumours, previously classified as leiomyomas, schwannomas or leiomyosarcomas, are today classified as GISTs on the basis of molecular and immunohistological features^[8]. GIST derives from the interstitial cells of Cajal (ICC), or from a common precursor of ICC and smooth muscle cells of the digestive

tract^[9]. Immunoperoxidase staining can show both c-kit and CD34-positive cells surrounding the Auerbach ganglia plexus in the gastrointestinal tract^[10]. The great majority of GISTs occur in the stomach (60%-70%) and small intestine (25%-35%)^[11]. In this study, 46.1% GISTs occurred in stomach and 23.1% in small intestine. In addition, the tumors arose in the esophagus, mesentery. The 52 cases of GISTs were subclassified as spindle or

epithelioid type stromal tumors based on the predominant pattern^[12]. Among them, spindle cell type was found in 20, epithelioid cell type in 20 and mixed type in 12.

To make a diagnosis of GISTs, immunohistochemical staining of the CD117 and CD34 is required, because they can characteristically express CD34 and CD117^[13]. In this study, CD117 and CD34 showed diffuse positive expressions, the positive rates were 98.1% and 92.3%. Eighteen cases with focal immunoreactivity for SMA were diagnosed as GISTs with smooth muscle differentiation. S-100, NSE, NF and MBP showed focal positive expressions, which could be used as the diagnostic criteria of GISTs with nerve differentiation.

As a sensitive and specific marker of GIST, *c-kit* seems to be a useful antibody in diagnosis and differential diagnosis of GIST, but it may not be used as a prognostic index^[14]. Coagulative necrosis, mitotic activity over 10/50HPF, high cellularity with obvious pleomorphism are also helpful parameters for diagnosis of malignancy aside from metastasis and invasion. Adhesion over 5 cm in diameter and mitotic activity over 5/50HPF but less than 10/50HPF might be the potentially malignant parameters^[15]. But the effective and reproducible diagnostic parameters for differentiating benign from malignant gastrointestinal stromal tumors (GISTs) are still not clear^[16].

p21WAF1 is a cyclin-dependent kinase inhibitor (CDKI) which contributes to the regulation of cell cycle progression by controlling CDK activity and induces a G1 arrest^[17,18]. Thus, it is a tumor suppressor gene and likely plays an important role in tumor development. Moreover, reduced expression of p21WAF1 has been reported to have a prognostic value in several human malignancies^[19]. Pindzola *et al*^[18] reported that malignant gastrointestinal stromal tumor expresses p21WAF1/CIP1. In this study, p21WAF1 expression was not associated with the localization, size and histological subtype of GISTs, except for the tumor grade showing a higher frequency of p21WAF1 expression in potential malignancy and malignancy than that in benign GISTs (75%, 85% and 5%, respectively), indicating that p21WAF1 expression plays an important role in potential malignancy and malignancy rather than in benign GISTs. However, there was no statistical significance between potentially malignant and malignant GISTs, suggesting that overexpression of p21WAF1 is associated with increasing malignant potential, and that p21WAF1 overexpression may be another useful marker in the assessment of the malignant potential in GIST.

The Bcl-2 protein family plays an important role in the regulation of apoptosis. This family contains both proapoptotic members (Bax, Bid, Bad, and Bak) and antiapoptotic members (Bcl-2 and Bcl-xl^[20]). Overexpression of Bax protein increases apoptosis^[21]. Previous studies have shown that Bax expression might be involved in differentiation/histological types of colorectal cancer^[22]. Chao *et al*^[23] reported that in endometrial carcinoma, the positive rate of Bax overexpression increases correspondingly with increase in histological grade. Although apoptosis is associated with the tumor grade of various carcinomas, little is understood about the association of apoptosis in mesenchymal tumors^[24].

Noguchi *et al*^[25] reported that there is no statistically significant difference in Bax expression between benign and malignant tumors. In our study, Bax expression was associated with tumor grade showing a higher frequency of Bax expression in potential malignancy and malignancy than in benign GISTs (91.7%, 80%, 10%, respectively), suggesting that Bax expression plays an important role in potential malignancy and malignancy rather than in benign GISTs. However, there was no statistical significance between potential malignancy and malignancy, suggesting that overexpression of Bax is associated with increasing malignant potential. Thus Bax overexpression may be another useful marker in assessment of the malignant potential in GISTs.

Yang *et al*^[26] reported that p21WAF1/CIP1 could inhibit proliferation and induce apoptosis of hepatocellular carcinoma cells, and that inhibition of VSMC growth by overexpression of human p21 gene is accompanied with induction of apoptosis. These results suggest that regulation of cell cycle by p21 may be closely linked to programmed cell death /apoptosis in human vascular smooth muscle cells^[27], but a number of recent studies have pointed out that in addition to being an inhibitor of cell proliferation, p21WAF1 acts as an inhibitor of apoptosis^[28,29]. In our study, a positive correlation was found between p21WAF1 and Bax ($r = 0.461$, $\kappa = 0.459$), demonstrating that p21WAF1 is closely linked to Bax. In conclusion, p21 gene induces apoptosis by increasing Bax expression and plays an important role in potential malignant and malignant GISTs. Moreover, other factors besides p21WAF1 may regulate Bax.

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BASIC RESEARCH

Formation of microchimerism in rat small bowel transplantation by splenocyte infusion

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CONCLUSION: Donor splenocyte infusion combined with CsA decreases remarkably the rejection and prolongs the survival time after rat small bowel transplantation.

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Key words: Microchimerism; Splenocyte infusion; Immunologic tolerance; Small bowel transplantation

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Abstract

AIM: To investigate the effect of donor splenocyte infusion combined with cyclosporine A (CsA) on rejection of rat small bowel transplantation (SBT).

METHODS: Male Sprague-Dawley (SD) rats and female Wistar rats weighing 230-270 g were used as donors and recipients respectively in the study. Heterotopic small bowel transplantation was performed. The rats were divided into three groups: group one receiving allotransplantation (SD→Wistar), group two receiving allotransplantation (SD→Wistar) + donor splenocyte infusion, group three receiving allotransplantation (SD→Wistar) + donor splenocyte infusion + CsA followed by CsA 10 mg/kg per day after transplantation, in which recipient Wistar rats were injected with 2×10^8 SD splenocytes 28 d before transplantation, and treated with CsA after transplantation. Finally, the specific DNA fragment of donor Y chromosome was detected in recipient peripheral blood and skin by PCR. The survival time after small bowel transplantation was observed. Gross and histopathological examinations were performed.

RESULTS: The survival time after small bowel transplantation was 7.1 ± 1.2 d in group 1, 18.4 ± 3.6 d in group 2 and 31.5 ± 3.1 d in group 3. The survival time was significant longer ($P < 0.01$) in group 3 than in groups 1 and 2. The gross and histopathological examination showed that the rejection degree in group 3 was lower than that in groups 1 and 2.

INTRODUCTION

Small bowel transplantation (SBT) has become an accepted therapy for intestinal diseases in patients who require total parenteral nutrition^[1,2]. Because of the rich lymphatic tissue in small bowel and its mesentery, the mesenteric lymph nodes and lymphatic plexus are transplanted along with the small bowel. Thus, small bowel transplantation has a more severe immune rejection compared with other organ transplantations^[3]. Immune rejection is the leading cause of failure in small bowel transplantation^[4-7]. Although the results of SBT have been dramatically improved during the past few years, the major impediment to success in SBT is still acute rejection (AR). The key steps toward a successful transplantation are therefore to attenuate immune reactions and to induce immune tolerance to grafts.

The spleen is an immunologically privileged organ. The incidence rate and degree of rejection after spleen transplantation are much lower than those of other solid organ transplantations. Splenocyte chimerism has been successful in inducing tolerance in acute and chronic rejection liver transplant models^[9]. Both in experimental study and in clinical practice, splenocytes (including lymphocytes, dendritic cells, Kupffer cells, *etc*) play an important role in immune tolerance induction. Although splenocyte chimerism can effectively decline the immune reactions in organ transplantation, but whether the administration of spleen cells to recipients has the same effect in SBT is uncertain. Some parameters were tested to confirm the anti-rejection effect of splenocyte infusion

combined with CsA on rat small bowel transplantation in this study.

MATERIALS AND METHODS

Animal preparation

Healthy inbred male Sprague-Dawley (SD) rats as donors, and inbred female Wistar rats as recipients, weighing 250 ± 20 g, provided by Medical Experiment Animal Center, Harbin Medical University, were housed in standard animal facilities, and fed with commercially available rat chow and tap water *ad libitum* for 1 wk before test to acclimatize to the laboratory. The donor and recipient were paired according to the similar body weight.

Rat small bowel transplantation

All procedures were performed under inhalation anesthesia with ether. The entire small bowel from the ligament of Treitz to the ileocecal valve was isolated with the superior mesenteric artery on a segment of aorta and portal vein^[14]. After donor systemic heparinization (300 U), the graft was perfused with 20 mL of cold lactated Ringer's solution *via* the aorta. The lumen was also washed in 20 mL of the same solution. In the recipient, end-to-side vascular anastomoses were performed between the graft aorta and recipient infra-renal aorta and between the graft portal vein and recipient inferior vena cava with 10-0 sutures using the standard microsurgical technique. The superior extremity of the transplanted small bowel was ligated and a distal small bowel stoma was performed on the left abdominal wall. Animals that died within 3 d were considered as technical failures and excluded from data collection^[10-12].

Spleen cell preparation

SD rats were sacrificed by decapitation. Spleens were collected and kept on ice in RPMI 1640 medium (Life Technologies, Grand Island, NY) supplemented with antibiotics (100 units/mL penicillin and 100 μ g/mL streptomycin). The spleens were disrupted in the medium by pressing spleen fragments between two glass slides. Cell suspensions were filtered through cotton gauze and washed three times with RPMI 1640 medium. Viable nucleated cells were counted and adjusted usually to 2.0×10^7 /mL.

Experimental groups and postoperative care

The rats were divided into three groups: group 1 as allotransplantation group (SD \rightarrow Wistar, $n = 10$), group 2 as allotransplantation (SD \rightarrow Wistar) + donor splenocyte infusion, group 3 as allotransplantation (SD \rightarrow Wistar) + donor splenocyte infusion + CsA, with CsA 10 mg/kg per day after transplantation, in which recipient Wistar rats were injected with 2×10^8 SD splenocytes 28 d before transplantation and treated with CsA after transplantation. Animals were fed with only sugar water (7 g/d) on d 1, rat chow and water on d 2 and thereafter. The rats' mental state, appetite, and ejection liquid of small bowel stoma were also observed.

Analysis of microchimerism

Microchimerism in peripheral blood and skin of Wistar rat recipients was assessed by PCR. Blood and skin were

collected, and genomic DNA was isolated from buffy coat with a DNA extract kit and from skin by the proteinase k-phenol-chloroform method. Microchimerism was detected by specific primers for the donor Y chromosome. PCR was performed with 100 ng of the DNA template, 18 pmol of forward (5'- CGT GGA GAG CGC AAG TT -3', p1) and reverse primers (5'- GTC GCT GTT TCT GCT GTA GTT A -3', p2). The primers were designed to distinguish donor DNA from recipient DNA, and yielded a visible PCR product of 154 bp with the donor DNA template under ethidium bromide fluorescence. The 50 μ L reaction contained 10 mmol/L Tris (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L MgCl₂, 0.001% gelatin (Perkin- Elmer, Foster City, CA), 50 g/L BSA, 0.2 mmol/L dNTPs, and 1 unit of Amplitaq DNA polymerase (Perkin-Elmer). The cycling conditions were: at 94°C for 4 min, followed by 35 cycles at 94°C for 60 s, at 55°C for 60 s, at 72°C for 60 s and a final extension at 72°C for 8 min in a GeneAmp PCR System 2400 (Perkin Elmer). The products were separated on 1.5% agarose gel containing ethidium bromide.

Histopathological analyses

Rats' small bowel allografts were excised from stoma or by laparotomy and fixed in 10% formalin. The fixed tissue was paraffin embedded, and tissue sections were stained with hematoxylin and eosin (HE). Rejection was graded histologically according to the phase of acute intestinal rejection established by Rosemurgy *et al*^[13] and Sudan *et al*^[14]. The sections were graded for tissue injury using a scale of 0 (none) to 7 (severe) based on the following criteria: 0: normal mucosa; 1: development of subepithelial (Gruenhagen's) spaces at villus tips; 2: extension of the subepithelial space with moderate epithelial cell lifting from the lamina propria; 3: massive lifting down sides of villi, some denuded tips; 4: denuded villi, dilated capillaries; 5: disintegration of the lamina propria; 6: crypt layer injury; 7: transmucosal infarction. All histological analyses were performed in a blinded fashion to avoid bias.

Graft survival

All recipients were followed up by visual inspection and submitted to autopsy soon after they died. Graft survival time was defined as the time until death of recipient due to immune rejection.

Statistical analysis

All data were analyzed by Student's *t* test and expressed as mean \pm SD. $P < 0.05$ was considered significant and $P < 0.01$ very significant.

RESULTS

Gross observation

The rats awaked soon after operation. In allotransplantation group (group 1), the rats presented various degrees of lethargy, anorexia hair disorder, unresponsiveness to outside stimulation and body weight loss. Intestinal graft was pale. Intestinal lumina was enlarged with massive adhesion and gradually aggravated, accompanying mass purulent discharge. Intestinal perforation occurred in some severe cases. In allotransplantation + donor splenocyte

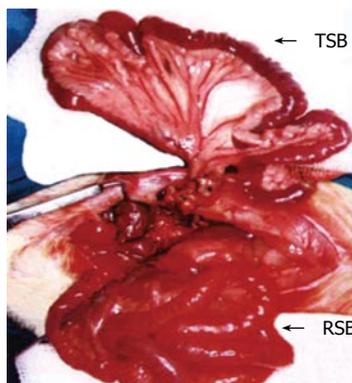


Figure 1 Heterotopic small bowel transplantation in rats. The graft vasculature was anastomosed. TSB: transplanted small bowel; RSB: recipient's small bowel.

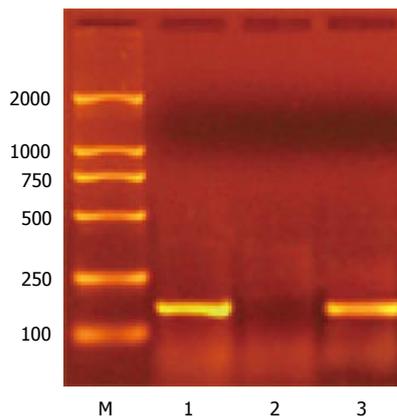


Figure 2 Agarose gel electrophoresis of PCR products. M: 2 Kb DNA ladder; lane 1: PCR amplification bands from normal SD rats; lane 2: PCR amplification bands from allotransplantation group rats; lane 3: PCR amplification bands from allotransplantation + splenocyte infusion group rats.

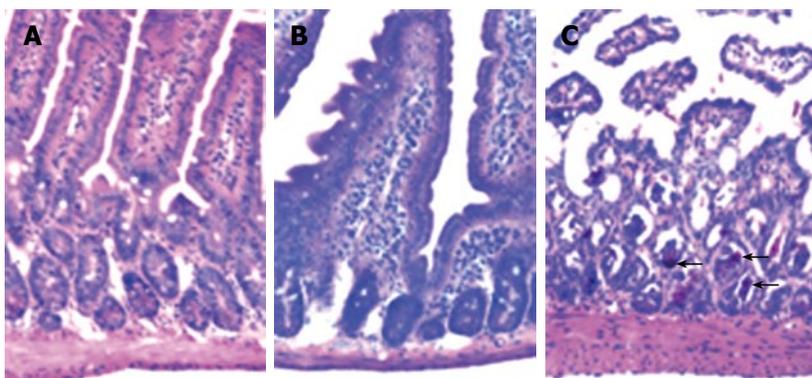


Figure 3 Histopathological findings in different groups after SBT. **A:** Few histopathological changes in the normal tissue (group 3); **B:** Intact villi and some infiltrates in allografts obtained from donor splenocyte recipients (group 2); **C:** Inflammatory infiltrates throughout the intestine and extensive apoptosis (arrows) and ulceration in the transplanted intestine (group 1).

Table 1 Survival time after small bowel transplantation in rats (mean \pm SD)

Group	<i>n</i>	Survival time (d)
group 1	10	7.1 \pm 1.2
group 2	10	18.4 \pm 3.6
group 3	10	31.5 \pm 3.1 ^b

^b*P* < 0.01 vs groups 1 and 2.

Table 2 Histologic grading of graft after small bowel transplantation in rats (mean \pm SD)

Group	<i>n</i>	Histologic grading
group 1	10	6.4 \pm 2.81
group 2	10	5.1 \pm 1.06
group 3	10	3.2 \pm 1.67 ^a

^a*P* < 0.05 vs groups 1 and 2.

infusion group (group 2), the rats were vigorous, sensitive to outside stimulation, and low-grade adhesion occurred 7 d after transplantation. In allotransplantation + donor splenocyte infusion + CsA group (Group 3), the rats were vigorous, and sensitive to outside stimulation, and low-grade adhesion occurred 6 d after transplantation (Figure 1).

Donor cell chimerism

Male donor cell chimerisms in recipients were determined by analyzing metaphase spreads for the presence of donor Y chromosomes. Y chromosomes were found in rats of the spleen cell infusion group but not in rats of allotransplantation group (Figure 2).

Survival time after transplantation

The survival time after small bowel transplantation was 7.1 \pm 1.2 d in group 1, 18.4 \pm 3.6 d in group 2, and 31.5 \pm 3.1 d in group 3. The survival time was significant longer (*P* < 0.01) in group 3 than in groups 1 and 2 (Table 1).

Histopathologic examination

Few histopathological changes and inflammatory infiltrate were detected in rats of group 3 (Figure 3A). After allotransplantation + splenocyte infusion treatment, some histopathological features of rejection were found, villi were intact but showed some blunting, crypts had no necrosis and lymphocyte infiltrate was minimal. A significant decrease in AR changes was observed in grafts of recipients treated with donor splenocyte infusion (Figure 3B). In group 1, severe rejection was characterized by complete villus flattening, epithelial apoptosis, and transmural cellular infiltrate (Figure 3C, Table 2).

DISCUSSION

In clinical practice, immune rejection induced by organ transplants necessitates the use of potent immunosuppressive drugs. However, excessive dosage of immunosuppressive agents may result in severe side effects, such

as hypertension, hepatic and/or renal toxicity. Moreover, prolonged usage of immunosuppressants often leads to severe infection and increased susceptibility to malignant tumors, thus critically affecting the health of recipients. It is therefore imperative to assess the protective effect of immune tolerance on organ transplantation. Recently, it has been reported that donor-derived multilineage hemopoietic cell microchimerism is a prerequisite for tolerance induction in organ allograft recipients^[15,16]. In an effort to augment the natural microchimerism that occurs following organ transplantation, adjunctive perioperative donor spleen cell infusion should be considered in conventionally immunosuppressed human organ transplant recipients^[17,18]. It was reported that donor spleen cell infusion can enhance liver allograft survival in humans^[19] because of the lack of recipient type antigen presenting cells (APC)^[20]. In our study, the survival time of the rats that received infusion of donor spleen cells combined with CsA was significantly longer compared to those that did not receive it.

The spleen is an immunologically privileged organ. The rejection incidence rate and degree of spleen transplantation are much lower than those of other solid organs. The spleen cell infusion can induce donor-specific transplantation tolerance and transient microchimerism. In our study, SD splenocyte chimerism was established in Wistar rats. SB grafts remained normal and were well vascularized. By contrast, the bowel of control animals showed AR.

The survival time was significant longer (31.5 ± 3.1 d *vs* 7.1 ± 1.2 d, $P < 0.01$) in group 3 than groups 1 and 2. The rejection degree in group 3 was lower than that in group 1. Rats in group 3 had better histological structures than rats in group 1 (3.2 ± 1.67 *vs* 6.4 ± 2.81 , $P < 0.05$), suggesting that donor spleen cell transfusion combined with cyclosporine A is a simple and practical method to suppress rejection of small bowel transplantation in clinical practice.

In conclusion, donor-specific tolerance induced by splenocyte chimerism prevents AR in an experimental model of highly immunogenic SB allografts. Central tolerance through splenocyte chimerism can be used to study the immune tolerance mechanisms. Further work is needed to reveal if chimerism is induced by spleen cell transfusion.

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CLINICAL RESEARCH

Hepaticojejunostomy for hepatolithiasis: A critical appraisal

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Abstract

AIM: To evaluate the long-term outcome and surgical indications of hepaticojejunostomy (HJ) for the treatment of hepatolithiasis.

METHODS: Three hundred and fourteen elective cases with hepatolithiasis but without biliary stricture or cystic dilatation treated in the past 10 years were reviewed retrospectively. The patients were divided into HJ group and T tube drainage group according to biliary drainage procedure. Furthermore, four subgroups were subdivided by hepatectomy as a balance factor, group A₁: hepatectomy+HJ; group A₂: choledochotomy+HJ; group B₁: hepatectomy + choledochotomy T tube drainage; group B₂: choledochotomy + T tube drainage. The stone residual rate, surgical efficacy and long-term outcome were compared among different procedures.

RESULTS: There was no surgical mortality among all patients. The total hospital mortality was 1.6%. The overall stone residual rate after surgical clearance was 25.9%. There was no statistical difference between HJ group and T tube drainage group in terms of stone residual rate after surgical clearance, however, after postoperative choledochoscopic lithotripsy, the total stone residual rate of T tube drainage group was significantly lower than that of HJ group (0.5% vs 16.7%, $P < 0.01$). Hepatectomy + choledochotomy tube drainage achieved the optimal therapeutic effect, only 8.2% patients suffered from an attack of cholangitis postoperatively, which was significantly lower than that of hepatectomy + HJ (8.2% vs 22.0%, $P = 0.034$). The major reason for postoperative cholangitis was stone residual in the HJ group (16/23, 70.0%), and stone recurrence in the T tube drainage group (34/35, 97.1%). The operative times were significantly prolonged in those undergoing HJ, and the operative morbidity of HJ was higher than those of T tube drainage.

CONCLUSION: The treatment result of HJ for hepatolithiasis is not satisfactory in this retrospective study due to high rate of stone residual and postoperative cholangitis. HJ could not drain residual stone effectively. HJ may hinder post-operative choledochoscopic lithotripsy, which is the optimal management for postoperative residual stone. The indications of HJ for hepatolithiasis should be strictly selected.

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Key words: Hepatolithiasis; Hepaticojejunostomy; Outcome

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INTRODUCTION

Hepatolithiasis is a common disease in Southeast Asia and is especially prevalent in China^[1,2]. This disease features a high stone residual and recurrence and its long-term outcome is far from satisfactory. The principles for the treatment of hepatolithiasis are complete clearance of stones, eradication of the diseased bile duct (stricture) and atrophic liver tissue and reconstruction of satisfactory bile drainage. Biliary-enteric anastomosis which mostly includes choledochoduodenostomy (CD) and Roux-en-Y hepaticojejunostomy(HJ), is one of the most common procedures used for hepatolithiasis. Due to the sump syndrome and high risk of stasis following CD^[3,4], many authors prefer HJ to CD as the standard procedure for benign biliary diseases^[4-6]. In fact, we have used HJ instead of CD to reconstruct enteric biliary drainage for hepatolithiasis or bile duct stricture in our department since 1990.

In the cases of hepatolithiasis simultaneously with extrahepatic bile duct stricture or congenital cyst dilatation, because the diseased bile duct should be resected, HJ is the treatment of choice to reconstruct bile drainage. However, in the cases without bile duct stricture or cystic dilatation, whether HJ is suitable remains controversial^[7-9].

In the current study, we reviewed the cases with hepatolithiasis treated surgically in our department in the past 10 years retrospectively, and evaluated the outcome and surgical indications of HJ for the treatment of hepatolithiasis.

MATERIALS AND METHODS

Patients

A total of 425 consecutive patients with hepatolithiasis treated surgically at the Department of Hepaticobiliary Surgery, the First Affiliated Hospital, Sun Yet-san University from June 1992 to June 2002 were reviewed retrospectively. According to our study purpose, the following cases were excluded because of no alternative surgical procedures for their conditions at that time, respectively, (1) 21 cases of hepatolithiasis complicated with acute obstructive suppurative cholangitis, who underwent choledochotomy, stone extraction and T tube drainage emergently once the diagnosis was confirmed; (2) 21 cases of hepatolithiasis with congenital choledochal cyst or Caroli's disease, who underwent stone removal, cyst resection or hepatectomy and HJ; (3) 23 cases of hepatolithiasis with left or right hepatic ducts or their second branches stricture, who received diseased bile duct resection and (or) bile duct stricturoplasty and HJ; (4) 46 cases of hepatolithiasis complicated with cholangiocarcinoma were also excluded. The remaining 314 elective cases were enrolled in this study, including 122 men, and 192 women. The mean age of whole group was 48 years (range: 15-88).

Grouping

According to the different procedures of bile drainage, 314 patients were divided into HJ group ($n = 123$) and T tube drainage group ($n = 191$). Because some cases in these two groups underwent hepatectomy simultaneously, we used hepatectomy as a balance factor to subdivide these two groups into four subgroups, Group A₁: Hepatectomy + HJ ($n = 76$); group A₂: choledochotomy + HJ ($n = 47$); group B₁: hepatectomy + choledochotomy T tube drainage ($n = 85$); group B₂: choledochotomy + T tube drainage ($n = 106$). The rate of residual stone, operative complications and therapeutic outcomes were compared among these groups.

Procedure of HJ

The standard procedure of HJ was an end-to-side, mucosa-to-mucosa anastomosis of the intra- and (or) extra-hepatic duct with a Roux-en-Y jejunal loop measuring 40 to 60 cm in length. The anastomosis was sutured with absorbable material (Viracyl, 4-0, Johnson Ltd, USA) interruptedly. A rubber tube measuring 3.5-5 mm in diameter was placed through biliary-enteric anastomosis retrogradely for later cholangiography or choledochoscopic manipulation. This drainage tube was removed when no stone resided within the biliary tract proofed by a cholangiogram at postoperative d 14. If there was residual stone proofed by cholangiography, the drainage tube tract was left for 6 wk to allow subsequent choledochoscopic manipulation. The indications of HJ we used previously were (1) the diameter of the common bile duct was larger than 2 cm; (2) intrahepatic stone located bilaterally; (3) peripheral bile duct stone that could not be cleared during surgery.

Measurement of residual stone

The diagnosis of postoperative stone residual was based on the cholangiogram performed through the T tube and

transanastomotic drainage tube at postoperative d 14 or ultrasound findings. Once residual stone was found, choledochoscopic stone extraction or lithotripsy (four-direction fiber choledochoscopy, 3.5 mm in diameter, Olympus Co., Japan) was done 6 wk after operation. This was repeated at 1 wk interval until the residual stone was completely cleared, or as clear as possible.

Postoperative follow-up

Follow-up was performed by reviewing medical records and patient interviews or telephone interview. At the end of this study, totally 241 out of 309 completed the follow-up (period: 2-12 years, median 7.6), the other patients were lost. Cholangitis was considered when patient presented with right upper quadrant pain, chill, fever or jaundice.

Statistical analysis

Patients' database was established by SPSS 11.0 software. *t*-test, rank test (continuous data) and Fisher's exact test, Chi-square test (categorical data) were used. $P < 0.05$ was considered statistical difference.

RESULTS

Operative mortality and hospital death

There was no surgical mortality in this study. Five cases (1.6%) died of hepatic failure postoperatively during their hospital stay, including 1 case in subgroup A₁, 2 cases in subgroup A₂, and 2 cases in subgroup B₂.

Patients' clinical characteristics

Patients' demographic and clinical data are shown in Table 1. The number of patients who had previous bile surgery of subgroups A₁ and A₂ was greater than those of subgroups B₁ and B₂, respectively ($P < 0.05$). The history and operating times of patients in the subgroups A₁ and A₂ were longer than those in subgroups B₁ and B₂, respectively ($P < 0.05$); the amount of blood loss in subgroups A₁ and A₂ was larger than those in the subgroups B₁ and B₂ respectively ($P < 0.05$).

Postoperative stone residual

The diagnosis of postoperative stone residual was based on the cholangiogram and ultrasound findings postoperatively. The most common site of stone residual was the intrahepatic duct. The overall stone residual rate after surgical clearance in our series was 25.9% (Table 2). There was no significant difference of stone residual between the HJ group and the T tube drainage group (20.3% vs 28.3%, $P > 0.05$). The cases who underwent hepatectomy [group (A₁ + B₁)] had less stone residual than those who did not, [group (A₂ + B₂)] ($P < 0.05$).

Postoperative complications

The common postoperative complications are listed in Table 3. The complications in subgroup A₁ was more than that in subgroup A₂ ($\chi^2 = 4.324$, $P < 0.05$).

Management of residual stone

The treatment of choice for postoperative residual stone

Table 1 Patients' demographic and clinical data

Groups	n	PS ¹ n (%)	Age (yr)	Hospital stay (d)	Operative time (min)	History ² (yr)	Bleeding ² (mL)
HJ group	123	90 (73.17)					
+hepatectomy (A1)	76	55 (72.37) ^a	45.4 ± 13.8	32.1 ± 12.0	282.9 ± 99.3 ^a	8.5 ^a (0.1-40)	500.0 ^a (150-27 000)
left lateral segmentectomy	52						
left hepatectomy	15						
right hepatectomy	3						
segmentectomy	6						
+CBD exploration (A2)	47	35 (74.47) ^c	51.8 ± 13.5	27.0 ± 8.5	226.1 ± 80.4	8.0 ^c (0.1-35)	300.0 (100-3000)
T tube drainage group	191	102 (53.40)					
+hepatectomy (B1)	85	46 (54.12)	46.6 ± 12.6	29.2 ± 10.6	189.4 ± 54.1 ^c	3.5 (0.1-40)	300.0 ^c (100-5000)
left lateral segmentectomy	57						
left hepatectomy	13						
right hepatectomy	4						
segmentectomy	11						
+ CBD exploration (B2)	106	56 (52.83)	49.8 ± 15.7	28.3 ± 16.6	166.4 ± 97.7	3.0 (0.1-40)	150.0 (30-18 000)

^a*P* < 0.05 vs group B1, ^c*P* < 0.05 vs group B2. ¹ps: previous bile surgery, ²expressed as median (range). Age, hospital stay and operative time were expressed as mean ± SD. CBD: common bile duct; HJ: hepaticojejunostomy.

Table 2 Sites and rates of residual stone

Groups	n	Site of stone residual					Rate Total (%)
		Left liver	Right liver	CBD	Left liver	Right liver	
HJ group	123	8	14	1	2	25	20.3 ^a
+hepatectomy (A1)	76	3	10	1	0	14	18.4
+CBD exploration (A2)	47	5	4	0	2	11	23.4
T tube drainage group	191	17	22	3	12	54	28.3
+ hepatectomy (B1)	85	5	10	1	2	18	21.2
+ CBD exploration (B2)	106	12	12	2	10	36	34.0

HJ, hepaticojejunostomy; CBD, common bile duct. ^a*P* > 0.05 vs T tube drainage group.

was choledochoscopic lithotripsy through the T tube or the trananastomotic drainage tube route 6 wk after operation. Totally 54 cases had residual stones in the T tube drainage group, and 53 had their stones completely cleared by choledochoscopic lithotripsy after 1 to 6 sections. Only 1 case was failed because his T tube withdrew simultaneously after discharge. However, only 5 cases whose residual stones could be successfully eliminated in HJ group, the other 20 cases failed because their fistula were unsuitable or difficult for the entry of choledochoscope, or the long Roux-en-Y loop hindered the technical manipulation. The overall stone residual rate of the T tube drainage group was significantly lower than the HJ group after choledochoscopic lithotripsy [0.5% (1/189) vs 16.7% (20/120), *P* < 0.01].

Postoperative cholangitis

Postoperative cholangitis presented by right upper quadrant pain, chill, fever and jaundice occurred at least once in 58 cases (Table 4). Five cases in the HJ group suffered from 4-6 episodes of cholangitis till the second operations were done. The main causes of cholangitis observed in our series were stricture of the biliary-enteric anastomosis (*n* = 3), stone residual (*n* = 16) or reflux of intestinal fluid (*n* = 1) in the HJ group, and stone recurrence (*n* = 34) or later

Table 3 Postoperative complications

Complications	Group A1 (<i>n</i> = 76)	Group A2 (<i>n</i> = 47)	Group B1 (<i>n</i> = 85)	Group B2 (<i>n</i> = 106)
Wound infection	6	3	5	7
Subphrenic collection/infection	7	0	3	0
Pleural infusion	3	0	2	0
Bile leakage	2	0	3	1
Biliary hemorrhage	1	0	0	2
Liver abscess	1	0	0	0
Liver failure	1	2	0	6
Pulmonary infection	0	1	1	1
Septicemia	1	0	0	0
Total	22	6	14	17
%	28.95 ^a	12.77	16.47	16.04

^a*P* < 0.05 vs group A2.

Table 4 Follow-up and postoperative cholangitis

Groups	n	Follow-up patients (<i>n</i> , %)	Postoperative cholangitis (<i>n</i> , %)	Causes of cholangitis			
				ST	SR	SRE	RC
HJ group	123	96 (78.0)	23 (24.0)	3	16	(3) ⁴	1
+ hepatectomy (A1)	76	59 (77.6)	13 (22.0)	1	9	3	0
+ CBD exploration (A2)	47	37 (78.7)	10 (27.0)	2	7	(2) ³	1
T tube drainage group	191	145 (76.0)	35 (24.1)	0	1	34	0
+ hepatectomy (B1)	85	61 (71.8)	5 (8.2) ¹	0	0	5	0
+ CBD exploration (B2)	106	84 (79.2)	30 (35.7) ²	0	1	29	0

¹*P* = 0.034 vs group A1. ²*P* = 0.349 vs group A2. ^{3,4}these two (or three patients) patients who had stone recurrence also had stricture of anastomosis. HJ: hepaticojejunostomy, ST: stricture of anastomosis or intrahepatic bile duct, SR: stone residual; SRE: stone recurrence; RC: reflux cholangitis.

development of bile stricture for chronic inflammation of bile duct in the T tube drainage group (Table 4).

DISCUSSION

Postoperative stone residual and recurrence still remain a

challenge in the treatment of hepatolithiasis. Though the systemic approaches have been used, the stone residual rate was over 30% in a nationwide survey of 4197 surgical hepatolithiasis cases in China^[2]. The overall stone residual rate after surgical clearance in our series was 25.9%. There was no significant difference between the HJ group and the T tube drainage group after surgical clearance. However, when hepatectomy as one of the treatments of choice was considered as a balance factor, we found that among patients who received hepatectomy the stone residual rate was significantly lower than those who did not. This finding is consistent with our previous report^[10] and other reports^[11-13].

Hepatolithiasis will cause bile duct stricture, liver parenchyma atrophy and chronic fibrosis due to repeated pyogenic cholangitis. These pathologic changes, especially bile duct stricture, may hinder stone extraction during surgery, even though the choledochoscopy was used. Hepatectomy provided the best therapeutic effect for completely removing the stone, the diseased bile duct (stricture ring) and atrophic liver parenchyma as well.

Postoperative choledochoscopic lithotripsy through the T tube or the bile duct drainage tube route is a potential remedial treatment for residual stone, as indicated by the T tube drainage group in this study. However, only 5 cases whose residual stone could be successfully removed by this method in the HJ group. Other cases with residual stone could not be removed due to the difficulty for choledochoscopy to access the bile duct through a long (40-60 cm) Roux-Y loop or because the tunnel was too small to enter for the choledochoscopy. After postoperative choledochoscopic lithotripsy, the overall stone residual in the T tube drainage group was markedly lower than that in the HJ group (0.5% *vs* 16.7%, $P < 0.01$). This indicates that HJ hinders postoperative choledochoscopic manipulation. For easy access to the biliary tract, hepaticocutaneous jejunostomy with a stoma after biliary surgery is recommended for the convenience of long-term treatment of stone residual or recurrence^[14]. However, hepaticocutaneous jejunostomy is not advocated for its possible complications related to fistula, infection, parastomal hernia, early stoma closure, and prolonged surgical procedure or hospitalization^[15].

Postoperative cholangitis is a critical factor to evaluate the long-term surgical result of this disease. Hwang *et al*^[16] reported that high risk factors related to cholangitis after initial surgery was bile duct stricture, residual stones, recurrent stones, and patients who were treated with nonhepatic resection. Our long-term follow-up (2-12 years, median 7.6) data demonstrated that patients who underwent hepatectomy with CBD exploration and T tube drainage had less attack of cholangitis, and the occurrence of cholangitis was significantly lower than those who underwent hepatectomy with HJ. Though the residual stones were nearly cleared in the T tube drainage group, and the immediate outcome was good, the occurrence of cholangitis was high. This was due to high recurrence of intrahepatic stone secondary to the bile duct stricture which was not treated during the initial surgery. The major reasons for postoperative cholangitis observed in our study were stone residual in the HJ group (16/23, 70.0%), and stone recurrence in the

T tube drainage group (34/35, 97.1%).

It was believed that the intrahepatic residual stone located in the peripheral bile duct would drain to Roux-Y loop through a large biliary-enteric anastomotic mouth simultaneously after surgery, therefore, residual stone would be cleared, and furthermore, HJ could prevent biliary-enteric regurgitation^[14,17]. To drain a residual stone, HJ is a popular procedure for hepatolithiasis, especially for the case with bilateral stones. Our data showed that the numbers of patients who had previous biliary surgery for hepatolithiasis were higher in the HJ group than that in the T tube drainage group. It suggests that HJ would be the final and optimal procedure for recurred or residual hepatolithiasis at that time. However, many clinical evidences showed that the intrahepatic residual stone could not drain to the Roux-Y loop completely and easily, especially for the stones located at the right posterior lobe and left lateral lobe of the liver. Rather they would accumulate continuously within the bile ducts, which was the main reason for the postoperative cholangitis episodes. This was also evidenced by the long-term follow-up data of the HJ group in this series. Actually, HJ can not completely block biliary-enteric regurgitation. The reflux cholangitis occurring in HJ performed for benign biliary diseases was 10%-15%^[18]. Besides the stone residual and bile duct stricture, postoperative cholangitis was also related to the decrease of motility of Roux-en-Y loop itself. Jejunal transection would lead to abnormal motility of the distal part of jejunum^[19,20]. Ducrotte *et al*^[21] reported that low motility of the Roux-en-Y loop presented by near-absence of phase III's activity, and an absence of response to meal was observed in recurrent cholangitis patients, and subsequently, microbes might colonize and overgrow in the loop. Later on they documented a similar result in asymptomatic patients undergoing HJ^[22].

HJ permanently eliminates the physiological preventive function of regurgitation by the papilla of Vater, which is a barrier between biliary tract and gastrointestinal tract, and subsequently, enteric biliary reflux and bacterial colonization of the biliary tract may occur^[23,24]. Furthermore, in a long-term large series study, Tocchi *et al* found that biliary tract may tend to develop malignancy following biliary-enteric bypass procedures for the benign biliary disease due to chronic inflammation of the bile duct^[25]. This indicates that bile duct cancer may be a long-term complication of biliary-enteric drainage.

On the other hand, our data also demonstrated that HJ prolonged the operation time and increased the risk of intraoperative bleeding and blood transfusion.

In conclusion, the overall long-term outcome of HJ for intrahepatic lithiasis was not satisfactory because of its high rate of stone residual and postoperative cholangitis in our study. HJ could not drain the residual stone effectively. Considering the shortcomings of HJ mentioned above, it is of critical importance to consider the surgical indications of HJ for hepatolithiasis. We advocate that HJ is needed only in the following conditions. (1) Hepatolithiasis complicated with extrahepatic ducts or its second branches stricture, which needs stricturoplasty and HJ. (2) Hepatolithiasis with congenital bile duct dilatation in which the dilated bile duct should be resected. (3) Dysfunction of the papilla of Vater, especially in the case of fibrotic

stricture of the papilla of Vater. Our results indicate that if intrahepatic stone could not be cleared during surgery, a T tube placement within the CBD rather than HJ would facilitate postoperative choledochoscopic lithotripsy.

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Differential treatment and early outcome in the interventional endoscopic management of pancreatic pseudocysts in 27 patients

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and an acceptable outcome with regard to the complication rate (11.1%) and mortality (3.7%), as shown by these initial study results.

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Abstract

AIM: Pancreatic pseudocysts (PPC) as a complication of pancreatitis are approached only in the case of abdominal pain, infection, bleeding, and compression onto the gastrointestinal tract or biliary tree.

METHODS: From 02/01/2002 to 05/31/2004, all consecutive patients with symptomatic PPC who underwent an interventional endoscopic approach were evaluated in this pilot case-series study: Group (Gr.) I-Primary percutaneous (external), ultrasound-guided drainage. Gr. II-Primary EUS-guided cystogastrostomy. Gr. III-EUS-guided cystogastrostomy including intracystic necrosectomy.

RESULTS: (=“follow up”: $n = 27$): Gr. I ($n = 9$; 33.3%): No complaints ($n = 3$); change of an external into an internal drainage ($n = 4$); complications: (a) bleeding ($n = 1$) followed by 3 d at ICU, discharge after 40 d; (b) septic shock ($n = 1$) followed by ICU and several laparotomies for programmed lavage and necrosectomy, death after 74 d. Gr. II ($n = 13$; 48.1%): No complaints ($n = 11$); external drainage ($n = 2$); complications/problems out of the 13 cases: 2nd separate pseudocyst ($n = 1$) with external drainage (since no communication with primary internal drainage); infection of the residual cyst ($n = 1$) + following external drainage; spontaneous PPC perforation ($n = 1$) + following closure of the opening of the cystogastrostomy using clips and subsequently ICU for 2 d. Gr. III ($n = 5$; 18.5%): No complaints in all patients, in average two endoscopic procedures required (range, 2-6).

CONCLUSION: Interventional endoscopic management of pancreatic pseudocysts is a reasonable alternative treatment option with low invasiveness compared to sur-

INTRODUCTION

Pancreatic pseudocysts (PPC) are complications of acute and chronic pancreatitis^[1-4], pancreatic trauma, and pancreatic duct obstruction^[4]. For differential diagnosis, PPC needs to be distinguished from cystic tumor lesions of the pancreas. An examination with transabdominal ultrasound and endoscopic ultrasonography (EUS) is considered an initial step of the diagnostic and therapeutic management. PPC are to be treated in the case of subsequent infection, bleeding, and compression onto the gastrointestinal tract or the biliary tree^[5]. Abdominal pain is the predominating clinical sign. Fever and septic symptoms are hints for infected PPC. The aim of the study was to demonstrate (1) the differential treatment strategies in PPC^[6], (2) the safety of an interventional endoscopic approach of PPC as a reasonable alternative and less invasive treatment option^[7,8], and (3) the outcome and short-term follow-up results including problems and complications^[7-9].

MATERIALS AND METHODS

From February 01, 2002 to May 31, 2004, all consecutive patients with symptomatic PPC were enrolled in this pilot case-series study. Patients underwent an interventional approach in the case of abdominal pain, fever, increase of C-reactive protein (CrP) above the normal range, and compression onto the gastrointestinal tract or the biliary tree caused by the PPC with

Table 1 Interventional endoscopic approach of PPC: Indications and methodological aspects of the 3 different treatment groups (see also "Patients and methods" section above)

GROUP	Title	n	Indication	Method
I	Primary, percutaneous, ultrasound-guided placement of a drainage	9	Septic symptoms & inhomogeneous cystic content with suspected infection of PPC revealed by transabdominal ultrasound & EUS	-Ultrasound-guided puncture of the PPC -Aspiration of 10-20 mL of cystic fluid/content -Laboratory analysis (amylase, lipase, CEA, Ca19-9) -Microbiologic investigation for microbial detection/growth -Placement of a 10-Fr. pigtail drainage (Endoflex, Voerde, Germany) -Rinsing with 20-50 mL NaCl/h (bolus) <i>via</i> the drainage -Administration of antibiotics (ceftriaxone [Rocephin [®] , Hoffmann-La Roche AG, Grenzach-Wyhlen, Germany] plus metronidazol [Clont [®] , Bayer Vital GmbH, Leverkusen, Germany]) over 7 d
II	Primary, EUS-guided cystogastrostomy	13	Echo-free PPC with no inner echos & no evidence of infection	-Puncture of the cyst with 19-G needle (Wilson-Cook [Cook Deutschland GmbH], Mönchengladbach, Germany) -Cytologic investigation of cystic fluid/content -Placement of a 8.5-Fr.-double pigtail catheter Endoflex, Voerde, Germany) -Administration of antibiotics (ceftriaxone [Rocephin [®] , Hoffmann-La Roche AG, Grenzach-Wyhlen, Germany]) over 3 d
III	EUS-guided cystogastrostomy with following necrosectomy	5	-Suspected sequester & necrosis within the PPC revealed by ultrasound -Persisting fever & septic symptoms after cystogastrostomy & EUS-guided placement of a drainage	-Introduction of a 0.035-Inch guide wire (MTW Endoskopie, Wesel, Germany) <i>via</i> needle in place -Opening of the PPC with needle knife (Erbe Elektromedizin GmbH, Leipzig, Germany) -Enlargement of the PPC opening with balloon dilatation (Boston Scientific Medizintechnik GmbH, Ratingen, Germany) -Endoscopy of the cystic cavity -Removal of the necroses with loop & Dormia's basket (MTW Endoskopie, Wesel, Germany, each) (min. 2x) -Microbiologic investigation for microbial detection/growth -Placement of a transgastrocystic 8.5-Fr.-double pigtail drainage (Endoflex, Voerde, Germany) -Removal of the pigtail catheter after 3-6 mo -Administration of antibiotics (ceftriaxone plus metronidazol) over 7 d

disturbance(s) of the gastrointestinal passage. Patients were subdivided into 3 groups as shown in Table 1.

A consent form was obtained from each patient enrolled in the study.

RESULTS

Overall, 27 consecutive patients with symptomatic PPC were enrolled in the study over the 28-mo study period. The following details comprise the outcome, subsequent therapeutic steps, and the follow-up data of the various treatment groups.

Group I (primary percutaneous, ultrasound-guided placement of a drainage): Nine patients of 27 (33.3%) underwent the approach of an external drainage of the PPC (Figure 1). In three patients, no further complaints were observed with no additional therapy. An internal drainage was placed in four patients (Figures 2 and 3). After the septic symptoms disappeared, the external drainage was replaced by an internal drainage (Figure 4). In the complication profile, there was one case of post-interventional bleeding with a subsequent 3-d stay at the ICU and discharge after a total of 40 d. In one case, septic shock occurred. The patient died after several open laparotomies for programmed lavages and necrectomy after a 74-d care at the ICU.

Group II (primary EUS-guided cystogastrostomy): By the mean of this approach, 13 of 27 patients (48.1%) were treated, aiming for the placement of an internal drainage for the PPC. In the vast majority of patients ($n = 11$),

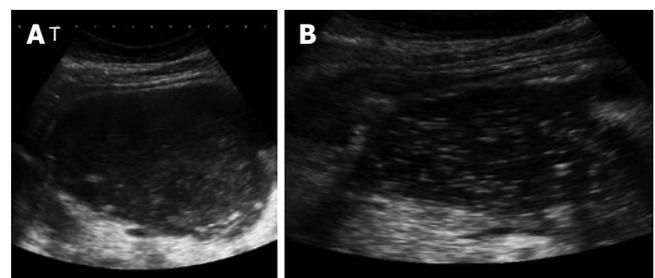


Figure 1 EUS images of an infected pancreatic pseudocyst (abscess) prior (A) and after placement (B) of an external drainage.

no further complaints or signs were reported, and no intervention-related symptoms were observed. Therefore, no further therapy was required. In two patients, an additional external drainage was placed: In one of the two patients, additional PPC were found, which, though communicating with other PPC, were not drained by the internally (transgastrocystically) placed drain reaching the originally detected PPC. In the second patient, a subsequent infection of the residual PPC was diagnosed following the initially successful internal drainage. This required a temporary external drainage. Out of 13 patients, there was only one serious complication. During the endoscopic intervention, a spontaneous perforation of the PPC was observed. Therefore, the cystogastrostomy was closed endoscopically by the mean of clips, which resulted in no further complaints, signs and symptoms after a 2-d stay and monitoring at the ICU. Hereafter, the patient's

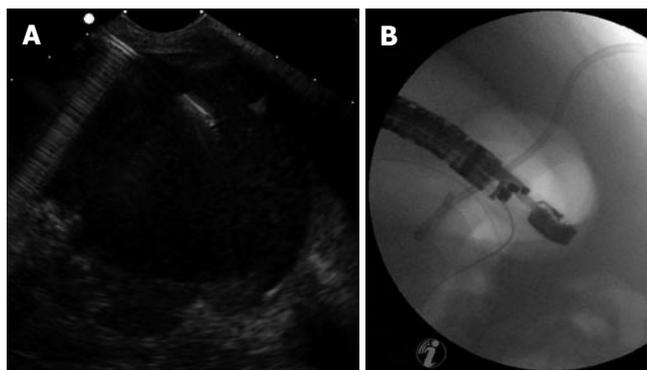


Figure 2 EUS-guided drainage of a pancreatic pseudocyst after previous external drainage: (A) EUS image; (B) Subsequent fluoroscopy control image of correct placement of the drainage.

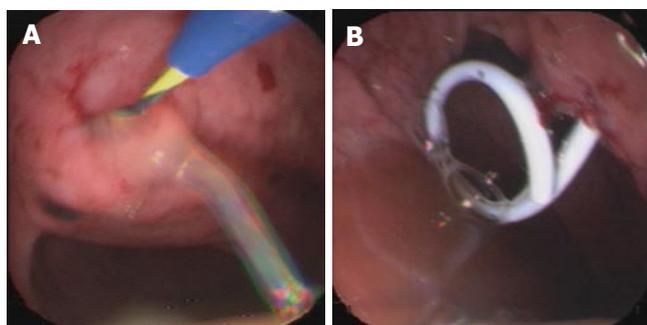


Figure 3 Endoscopy-guided cystogastrostomy via guide wire (A) and placement of an internal drainage (B).

discharge became possible.

Group III (EUS-guided cystogastrostomy including necrosectomy): This approach was used successfully in 5 of 27 patients (18.5 %) (Figure 5). The postinterventional course of all patients was uneventful and they did not show any problems or complications. On average, two endoscopic procedures (range, 2-6) were required to achieve the intended aim.

All together, there were three serious complications out of 27 subjects who underwent an interventional endoscopic approach of their PPC resulting in a complication rate of 11.1%. However, one patient died post-interventionally (mortality, 3.7%) because of septic complications with the underlying necrotizing pancreatitis.

DISCUSSION

In this report, treatment results for a novel interventional endoscopic approach for PPC are described. According to the aim of the study, this approach is a reasonable initial option in the therapeutic spectrum and it can be considered an establishing, less invasive, feasible, safe, and effective treatment alternative in experienced hands compared with open surgery, showing acceptable outcome and follow-up results with regard to complication rate and mortality^[1-4,9-12]. The great advantage of this endoscopic approach is that it avoids the more severe trauma and invasiveness of a surgical intervention (no general

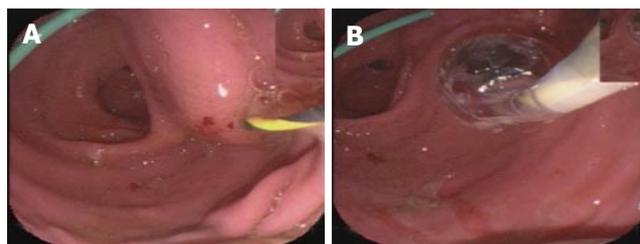


Figure 4 Endoscopic images: (A) EUS-guided puncture after previous external drainage; (B) Balloon dilatation via guide wire.

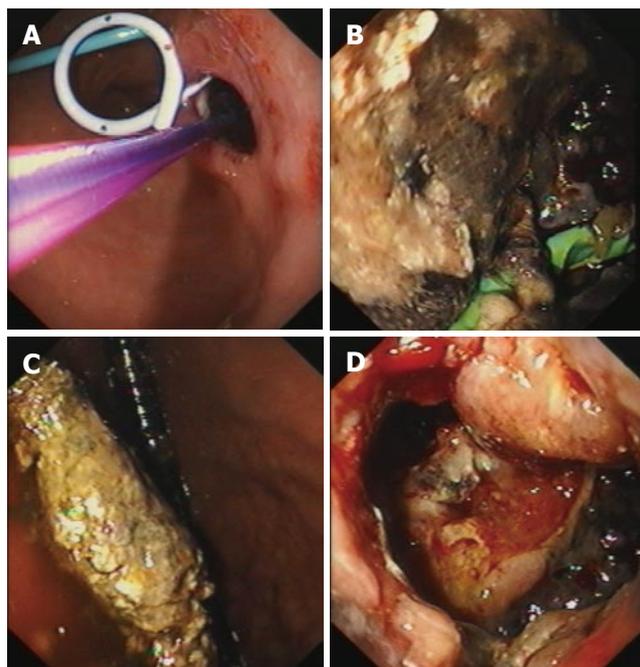


Figure 5 Endoscopic view: (A) After EUS-guided placement of an internal drainage (external drainage previously); (B) Into the pseudocystic cavity showing necroses; (C) Showing necrosectomy; (D) Through the transgastrocystic opening into the necrosectomized pseudocystic cavity.

anesthesia, no laparotomy, no risk for wound infection, less alteration of pulmonary and cardiac function, benefit for high-risk patients with a number of accompanying diseases or significant problems in their medical history). This endoscopic approach keeps the surgical intervention option still available in the case of unsuccessful outcome and follow-up. The endoscopic approach may provide comparable complication and mortality rates as shown for open cystogastrostomy or cystojejunostomy in a reasonably selected group of patients. However, the endoscopic approach has not changed but emphasizes the indications and principles^[6] for the interventional treatment of PPC as follows: (1) Only symptomatic PPC are to be treated^[6,12], (2) Basically, decision-making favors a forced interventional endoscopic approach together with an abdominal surgeon for surgical back-up^[6]. (3) EUS is the main tool for diagnostic and treatment of PPC^[3,4,6,10,13,14].

In particular, the novel aspect of the suggested approach is the less invasive character of this type of endoscopic intervention. The endoscopic approach

includes differential treatment options (primary percutaneous, ultrasound-guided placement of a drainage EUS-guided cystogastrostomy with or without subsequent necrosectomy) and a comparable outcome to open surgery as indicated by peri-interventional morbidity and mortality.

The main complications and problems of the endoscopic procedure are subsequent infection of the cyst^[6-9], bleeding^[3,6-10], pancreatitis^[7,8], perforation^[9], colonic fistula^[15], failure of the drainage^[2], conversion^[1], PPC recurrence^[2,9,10,12], and even death. In our study, we observed only one bleeding, one perforation and one case of septic shock but none of the other possible complications. We showed a complication rate of 11.1% ($n = 3$), which is relatively low compared to De Palma *et al*^[7,8] who reported a complication rate of 24.5%. In our study, there was one death due to necrotizing pancreatitis possibly indicating an unsuitable case for the endoscopic procedure.

According to this study, we suggest the following algorithm (a modification of Vosoghi^[4] and Baillie^[6]): (1) Initially, a possible malignant tumor growth of the cystic lesion needs to be excluded by means of EUS^[6], laboratory analysis of cystic contents and cytologic investigation. In the case of an echogenic, thickened cystic wall and detectable Doppler signals within the cystic septum^[6], a malignant cystic tumor lesion can be suspected. The suspected diagnosis can be supported by cytologic investigation of cystic content detecting tumor cells. In addition, levels of tumor markers such as CEA and Ca19-9 can be analyzed in the cystic fluid. The sensitivity and specificity of Ca19-9 to predict malignant tumor growth in cystic lesions of the pancreas is approximately 80% (cut off point, 50 000 IU/mL). In the case of a malignant cystic tumor lesion, a surgical approach is obligatory. If there are no hints of malignancy, (2) ERCP follows to clarify whether the PPC communicates with the pancreatic duct^[6]. If yes, (3a) a transpapillary drainage needs to be implanted^[4,6-10]. Otherwise, (3b) a transgastrocystic, EUS-guided drainage is placed^[4,6-10]. If there are hints of an infected PPC or an abscess (echo-rich cystic content, necroses, sequester) revealed by transabdominal ultrasound, EUS, or clinical exam, primarily, (3c) an external (percutaneous) drainage of the PPC is to be favored^[2,4]. After placement of a drainage, rinsing the cyst over several days and administration of antibiotics can be initiated. Depending on the patient's clinical course (persisting PPC of the same size and configuration, persisting septic signs and symptoms, no falling inflammatory laboratory parameters) it has to be decided whether additionally, (3d) a necrosectomy^[6] or even (3e) surgical intervention is required^[4].

An interesting additional approach to consider is a laparoscopic endogastric pseudocyst gastrostomy, which combines less invasiveness, in particular, in large retrogastric PPC with the potential option of facilitated debridement of necrotic pancreas^[1,2].

In conclusion, interventional endoscopic management of pancreatic pseudocysts is a reasonable alternative treatment option considering: (1) surgical principles^[6] and (2) lower invasiveness.

Compared with surgical intervention there is a similar or even lower complication rate and mortality as shown by these study results. Subsequent follow-up investigations of the patients treated in this report, favoring again EUS, and studies with a higher value of evidence and larger case numbers are necessary to investigate treatment outcome and possible long-term consequences more appropriately^[2,11].

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Prevalence of factor V Leiden and prothrombin G20210A in patients with gastric cancer

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Abstract

AIM: To analyze the prevalence of the two commonest thrombophilic mutations, factor V Leiden and prothrombin G20210A, in patients with gastric cancer.

METHODS: One hundred and twenty-one patients with primary gastric carcinoma and 130 healthy subjects, comparable for age and sex, were investigated. Factor V Leiden was detected by using polymerase chain reaction and restriction enzyme digestion, and prothrombin G20210A gene mutation by allele-specific PCR.

RESULTS: Among the 121 cancer patients, factor V Leiden was found in 4 cases (GA genotype: 3.3%) and prothrombin G20210A in 10 cases (GA genotype: 8.3%). Of the 130 control subjects, factor V Leiden was detected in 6 cases (GA genotype: 4.6%) and prothrombin G20210A in 8 cases (GA genotype: 6.1%). No double heterozygous carriers of both mutations were found in either group. The prevalence of both factor V Leiden and prothrombin G20210A variant was not statistically different between the cancer patients and the healthy subjects.

CONCLUSION: Our study suggests that, in gastric cancer, the risk factors of thrombophilic cancer state are on acquired rather than on a genetic basis and that prothrombin G20210A does not seem to be a cofactor in gastric cancer pathogenesis.

INTRODUCTION

The pathogenesis of haemostatic disorders in cancer is complex and mainly on acquired basis. To date, little conclusive information is available in literature about the association of cancer hypercoagulability and inherited thrombophilia. The most common genetic defect in Caucasian population is factor V Leiden, a glutamine to arginine switch at amino-acid 506, due to G to A transition at nucleotide 1691 of coagulation factor V. This point mutation makes factor Va resistant to the proteolytic action of activated protein C so that activated factor V persists, rather than being inactivated. Another genetic defect is prothrombin G20210A polymorphism, a G to A transition at nucleotide 20210 in the 3'-untranslated region of the prothrombin gene, associated with elevated levels of prothrombin, which contributes to thrombotic risk by promoting enhanced thrombin generation.

Recently, Miller *et al*^[1] found an increased incidence of neoplasia of the digestive tract in men with persistent activation of the coagulation pathway. In addition, there is some evidence that prothrombin G20210A gene mutation may be involved in cancer development and/or progression. The prevalence of factor V Leiden and prothrombin G20210A gene polymorphism was analyzed in a cohort of 175 patients with gastrointestinal carcinoma and a significantly increased prevalence of prothrombin gene mutation in the patient group as compared to normal controls was detected^[2]. On the contrary, Paspatis *et al*^[3] demonstrated that the prevalence of both factor V Leiden and prothrombin G20210A in 74 colorectal cancer patients was found to be similar to that of 192 colonoscopically selected control subjects. To the best of our knowledge, no data regarding the prevalence of factor V Leiden and prothrombin G20210A in the subset of patients with gastric cancer are available in the literature. We, therefore, performed a prospective case-control study to analyze the

prevalence of factor V Leiden and prothrombin G20210A in patients with gastric cancer.

MATERIALS AND METHODS

One hundred and twenty-one consecutive patients (78 men, 43 women; mean age: 62 years, range: 51-76 years) with operable gastric carcinoma (TNM staging: T₁₋₃, N₀₋₂, M₀) and 130 healthy subjects, matched for age, sex and ethnic-background, were investigated for the presence of factor V Leiden and prothrombin G20210A gene mutation. All individuals were from central Italy, without any previous thrombotic event. Specifically, no control subject or cancer patient had a history of peptic ulcer or *H pylori* infection.

Genomic DNA was extracted from white blood cells according to standard procedures. Factor V Leiden was detected by using polymerase chain reaction and restriction enzyme digestion following the methods described by Bertina *et al*^[4]. Prothrombin G20210A gene mutation was detected by allele-specific PCR according to the methods described by Poort *et al*^[5].

Statistical analysis

Statistical analysis was performed using χ^2 test (with Yates correction).

RESULTS

Among the 121 cancer patients, factor V Leiden was found in 4 cases (GA genotype: 3.3%) and prothrombin G20210A variant in 10 cases (GA genotype: 8.3%). Among the 130 control subjects, factor V Leiden was detected in 6 cases (GA genotype: 4.6%) and prothrombin G20210A variant in 8 cases (GA genotype: 6.1%). No double heterozygous carriers of both mutations were found in both groups. The prevalence of both factor V Leiden and prothrombin G20210A was not statistically different between the cancer patients and the healthy subjects.

DISCUSSION

The present study suggests that, in gastric cancer patients, there is no increase in the prevalence of both fac-

tor V Leiden and prothrombin G20210A gene mutation. These data seem apparently in contrast with the results of Pihusch *et al*^[1], obtained in a cohort of patients with carcinoma of all the gastrointestinal tract (most patients with adenocarcinoma of the colon), and this could indicate a different, peculiar pathogenetic pathway of gastric carcinogenesis. To our knowledge, this is the first report about the prevalence of factor V Leiden and prothrombin G20210A in a cohort of gastric cancer patients. Moreover, the ethnic background of our subjects is different and this could partly explain our results. On the other hand, our data are in agreement with the findings by Paspatis *et al*^[2] who did not find a significant difference in the prevalence of the two prothrombotic polymorphisms in 74 colorectal cancer patients as compared to the controls. However, the limitation of our study is the low statistical power of our sample size.

In conclusion, our data strengthened the evidence that, in gastric cancer, the thrombophilic state is on acquired rather than on genetic basis and seem to suggest that the prothrombin G20210A is not involved in gastric cancer pathogenesis.

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Mass-forming pancreatitis: Value of contrast-enhanced ultrasonography

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Abstract

AIM: To assess the utility of contrast-enhanced ultrasonography (CEUS) with a second-generation contrast medium in the differential diagnosis between mass-forming pancreatitis and pancreatic carcinoma.

METHODS: From our radio-pathology database, we retrieved all the patients affected by mass-forming pancreatitis or pancreatic carcinoma who underwent CEUS. We evaluated the results of CEUS in the study of the 173 pancreatic masses considering the possibilities of a differential diagnosis between mass-forming pancreatitis and pancreatic tumor by identifying the "parenchymographic" enhancement during the dynamic phase of CEUS, which was considered diagnostic for mass-forming pancreatitis.

RESULTS: At CEUS, 94% of the mass-forming pancreatitis showed intralesional parenchymography. CEUS allowed diagnosis of mass-forming pancreatitis with sensitivity of 88.6%, specificity of 97.8%, positive predictive value of 91.2%, negative predictive value of 97.1%, and overall accuracy of 96%. CEUS significantly increased the diagnostic confidence in the differential diagnosis between mass-forming pancreatitis and pancreatic carcinoma, with receiver operating characteristic curve areas from 0.557 ($P = 0.1608$) for baseline US to 0.956 ($P < 0.0001$) for CEUS.

CONCLUSION: CEUS allowed diagnosis of mass-forming pancreatitis with diagnostic accuracy of 96%. CEUS significantly increases the diagnostic confidence with respect to basal US in discerning mass-forming pancreatitis from pancreatic neoplasm.

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Key words: Contrast-enhanced ultrasonography; Pancreatitis;

INTRODUCTION

Mass-forming pancreatitis usually arises in patients with a history of chronic pancreatitis^[1]. The main characteristic feature at pathology is progressive interstitial fibrosis with chronic inflammatory infiltrate. Differential diagnosis with a neoplastic disease may be difficult because mass-forming pancreatitis and pancreatic tumor may present with the same symptoms and signs^[2]. Autoimmune pancreatitis is a rare type of chronic pancreatitis which has been proposed as a separate clinical entity in 1995 and later defined^[3,4]. As opposed to the other forms of chronic pancreatitis, in the autoimmune form, the pancreas is increased in volume, usually in a diffuse way with the typical "sausage" look, and Wirsung duct is compressed by glandular parenchyma or string-like^[4]. At ultrasonography (US), mass-forming pancreatitis is often very similar to pancreatic carcinoma^[1,5], presenting in most cases as a hypoechoic mass in a limited sector of the gland, usually at the head, often with enlargement or lumpiness of the gland contour. Contrast-enhanced ultrasonography (CEUS), thanks to the real-time continuous visualization of blood perfusion of the pancreas and its masses, has been recently used in the evaluation of the vascularization of solid pancreatic lesions, with results superior to single-slice spiral CT^[6,7]. The CEUS features of autoimmune pancreatitis have also been evaluated^[8]. In this study, we aimed to assess the utility of CEUS with a second-generation contrast medium in the differential diagnosis between mass-forming pancreatitis and pancreatic carcinoma.

MATERIALS AND METHODS

From our radio-pathology database, we retrieved all the patients affected by mass-forming pancreatitis or pancreatic ductal adenocarcinoma who underwent CEUS at our Institution between January 2002 and January 2005. Our Institutional Review Board does not require any informed consent for retrospective studies. This study included 35

patients (26 males, 9 females, mean age 49.1 years) affected by mass-forming pancreatitis (19 chronic alcohol-related pancreatitis, 15 chronic autoimmune pancreatitis, 1 genetic pancreatitis) and 138 patients affected by pancreatic tumors (78 males, 60 females, mean age 62.4 years). A total of 173 pancreatic masses were enrolled. All the pancreatic masses underwent cytological or histological diagnosis. All the patients with cytological diagnosis of pancreatitis were followed up at least for one year. All CEUS examinations were performed by radiologists on a Sequoia 512 6.0 (Acuson, Mountain View, CA, USA) ultrasound system, with harmonic microbubble-specific imaging with low acoustic ultrasound pressure (2-4 MHz Coherent Contrast Imaging or Cadence Contrast Pulse Sequencing; Mechanical Index 0.2; 12-13 frames/s). A 2.4 mL bolus of a second-generation contrast medium, SonoVue® (Bracco, Milan, Italy), was intravenously injected, followed by a 5 mL bolus of saline solution. All CEUS examinations were performed by the same radiologist and recorded on videotape/VHS or magneto optical disk/MOD systems to have the possibility to immediately review the dynamic study. Insonation of the pancreatic lesion was continuous with dynamic observation of the shift from the unenhanced phase to the contrast-enhanced phase. The enhancement pattern of the lesions was compared to that of the adjacent normal parenchyma. Definition of the arterial phase is possible when observing hyperechogenicity of the aorta or other big perilesional arteries. The venous phase is defined when the splenomesenteric-portal tree becomes hyperechoic. The lesions were classified according to the lesional enhancement in the enhanced phases as hypovascular/hypoechoic (lesions almost without enhancement or with enhancement lower than that of the adjacent parenchyma), isovascular/isoechoic (lesions with slight continuous enhancement or enhancement similar to that of the adjacent parenchyma) and hypervascular/hyperechoic (lesions with bright enhancement or enhancement superior to that of the adjacent parenchyma). The presence of a slight continuous enhancement inside the pancreatic masses, isovascular with the adjacent pancreatic parenchyma, was defined as "parenchymographic enhancement". We evaluated the results of CEUS in the study of the 173 pancreatic masses considering the possibilities of a differential diagnosis between mass-forming pancreatitis and pancreatic tumor by identifying the parenchymographic enhancement during the dynamic phase of CEUS, which was considered diagnostic for mass-forming pancreatitis. The reports of all the CEUS examinations were reviewed retrospectively, but all the CEUS studies had been interpreted in a prospective manner by the attending radiologist and so utilized for the data analysis. The sensitivity, specificity, positive and negative predictive values and diagnostic accuracy of CEUS in the characterization of the pancreatic masses were then calculated. Moreover, to compare baseline US and CEUS, each mass-forming pancreatitis was evaluated at baseline US and at CEUS with a 3 level diagnostic score: 0 = absence of malignancy (isoechoic lesions); 1 = indeterminate (hyperechoic lesions); and 2 = presence of malignancy (hypoechoic lesions). Diagnostic confidences of baseline US and of CEUS in the characterization of mass-forming

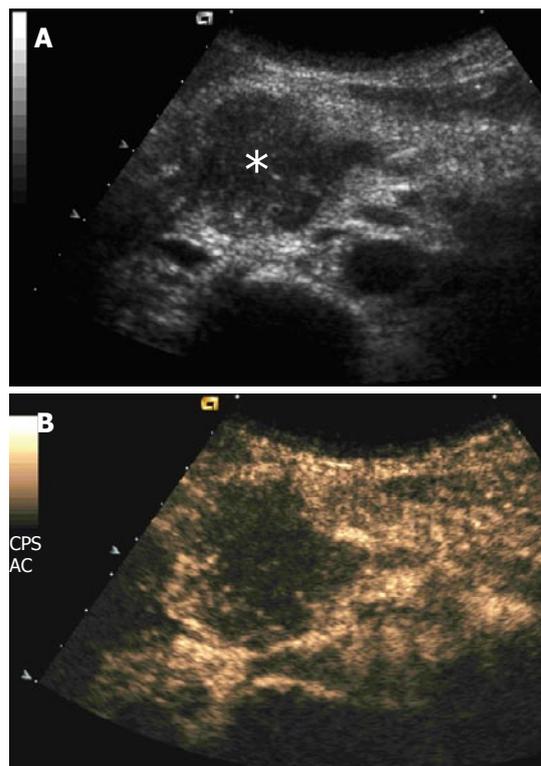


Figure 1 Ductal adenocarcinoma. **A:** US showing slightly hypoechoic pancreatic head mass (asterisk); **B:** CEUS showing poor enhancement of the mass, appearing hypoechoic to the rest of pancreatic parenchyma in the contrast-enhanced phases.

pancreatitis were represented by means of the ROC (receiver operating characteristic) curves; diagnostic advantage of CEUS on baseline US was calculated by comparing the areas under the ROC curves obtained by using a computer software package (Analise-it; Analise-it-software, Leeds, England).

RESULTS

All the contrast-enhanced ultrasound examinations were technically adequate allowing the dynamic observation of the shift from the unenhanced to the contrast-enhanced arterial and venous phases.

Pancreatic tumors

Pancreatic carcinomas were hypoechoic to the adjacent parenchyma at CEUS during the dynamic phases in 91% (126/138) of the cases (Figure 1), while hyperechoic and isoechoic in 7% (9/138) and 2% (3/138) of the cases, respectively.

Mass-forming pancreatitis

Mass-forming pancreatitis involved diffusely the pancreatic parenchyma in 8.5% (10/35) of the cases, while 71.5% (25/35) of the cases were focally localized. Of the 25 focally localized cases, 16 were at the pancreatic head, 5 at the uncinate process and 4 at the pancreatic body. The main pancreatic duct was dilated in 10 cases, the common bile duct was dilated in 11, and dilation of both ducts was observed in 6 cases. Lesional calcifications were

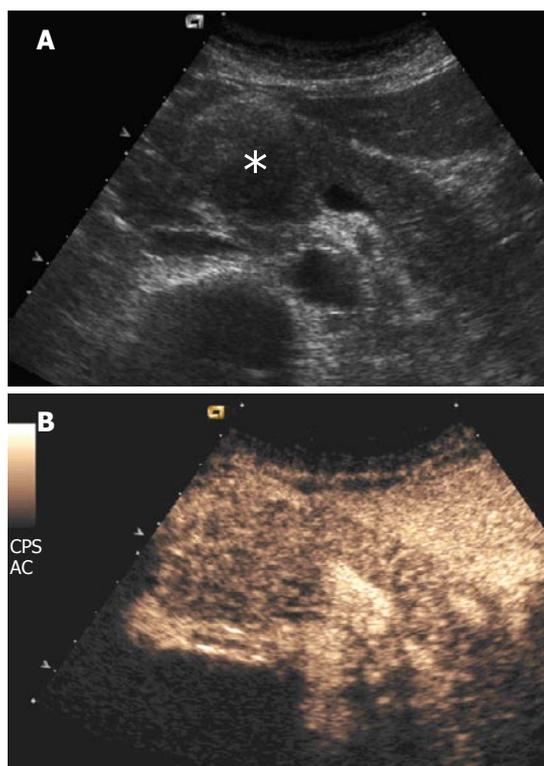


Figure 2 Mass-forming chronic autoimmune pancreatitis. **A:** US showing hypoechoic head pancreatic mass (asterisk); **B:** CEUS showing parenchymographic enhancement of the pancreatic lesion in the head of the pancreas during the contrast-enhanced phases.

observed in 5 (14%) patients. One patient was positive for the SPINK-1 genetic marker. Five patients had significantly higher levels of serum tumoral markers (CA 125 in 1 and CA 19-9 in 4 cases). At CEUS, 94% of the mass-forming pancreatitis showed intralesional glandular parenchymography (Figure 2), while 6% of the mass-forming pancreatitis remained hypoechoic during the dynamic phase. CEUS allowed diagnosis of mass-forming pancreatitis, assuming isoechogenicity as significant for pancreatitis, with sensitivity of 88.6%, specificity of 97.8%, positive predictive value of 91.2%, negative predictive value of 97.1%, and overall accuracy of 96%. The presence of parenchymographic enhancement or hypoechogenicity in the examined pancreatic masses in the dynamic phases of CEUS significantly increased the diagnostic confidence in the differential diagnosis between mass-forming pancreatitis and pancreatic tumor, with receiver operating characteristic curve areas from 0.557 ($P = 0.1608$) for baseline US to 0.956 ($P < 0.0001$) for CEUS (Figure 3).

DISCUSSION

Mass-forming pancreatitis is caused by various etiopathogenetic factors. However, at least two distinct categories have been recognized: alcohol-induced and autoimmune-related^[9]. Differential diagnosis between mass-forming pancreatitis and pancreatic tumor is a crucial point for the correct management of patients affected by pancreatic masses. However, differential diagnosis can be difficult in

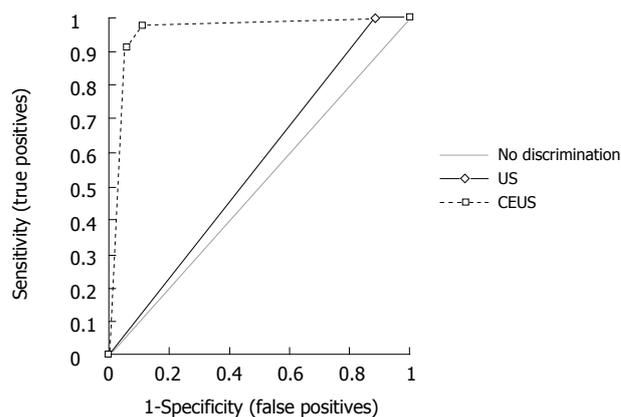


Figure 3 Receiver operating characteristic curves of baseline ultrasound and contrast-enhanced ultrasound in the characterization of 173 pancreatic masses, discerning between benignancy (pancreatitis) and malignancy (tumor).

clinical practice^[10,11]. In fact, mass-forming pancreatitis and pancreatic tumors may present with the same symptoms and signs^[2]. Several clinical and imaging features have been proposed to be helpful for the differential diagnosis. In case of mass-forming chronic pancreatitis, the presence of small calcifications in the lesion may suggest its inflammatory nature^[12], but is surely poorly specific. The identification of perfusion features similar to those of the normal pancreatic parenchyma (i.e., glandular parenchymography) is strongly suggestive of inflammatory mass, while ductal carcinomas typically display a low/absent enhancement due to the scirrhous content of this tumor. Contrast-enhanced ultrasonography (CEUS) has recently been used for pancreatic lesions study with good results^[6,13]. The CEUS finding consistent with an inflammatory origin of a pancreatic mass is the presence of a parenchymographic enhancement, defined as slight continuous enhancement inside the pancreatic mass with isovascularity to the adjacent pancreatic parenchyma. In this study, considering the presence of this sign, CEUS diagnosis of mass-forming pancreatitis was possible with sensitivity of 88.6%, specificity of 97.8%, positive predictive value of 91.2%, negative predictive value of 97.1% and an overall diagnostic accuracy of 96%. The intensity of this parenchymographic enhancement is surely related to the length of the underlying inflammatory process. It has been observed that the more the inflammatory process is chronic and long-standing, the less intense is the intralesional parenchymography, probably in relation to the entity of the associated fibrosis. As opposed to this, in mass-forming pancreatitis of more recent onset, the enhancement is usually more intense and prolonged^[5]. The characteristic findings of autoimmune pancreatitis have been well defined: increased levels of serum gammaglobulin or IgG; presence of autoantibodies; enlargement of the pancreas; diffusely irregular narrowing of the main pancreatic duct and occasional stenosis of the intrapancreatic bile duct; fibrotic changes with lymphocyte infiltration; absent or mild symptoms; rare pancreatic calcifications and cysts; occasional association with other autoimmune diseases and effective steroid therapy^[14]. Autoimmune pancreatitis is also reported to be characterized

by periductal fibrosis, mainly sustained by lymphocytic infiltration, with evolution to fibrosis^[3,4]. The exact incidence and prevalence of this disease are not known, but previous studies have shown a male preponderance (ratio of 2:1) and a predominant involvement of the elderly age group^[15]. The association with other autoimmune diseases has been reported, although data about the exact incidence of this association are not available^[14]; the most commonly associated diseases are Sjogren syndrome, diabetes mellitus, inflammatory bowel disease (especially Crohn's disease), primary biliary cirrhosis, primary sclerosing cholangitis and systemic lupus erythematosus^[15,16]. Response to steroid therapy is reported in the literature, but dosage and duration of the therapy are not standardized. However, pancreatitis is the most common benign condition that mimics pancreatic neoplasm, and in recent experience at Johns Hopkins, lymphoplasmacytic sclerosing pancreatitis is the most common form of pancreatitis in patients who are subjected to pancreaticoduodenectomy for suspected neoplasm^[17]. Ultrasonographic features of autoimmune pancreatitis are very similar to those of focal pancreatitis, even though autoimmune pancreatitis may interest more frequently the entire gland or present a larger extension and ubiquitous localization. US findings are characteristic in the diffuse form when the entire gland is involved. Echogenicity is markedly reduced, gland volume is increased and Wirsung duct is compressed by glandular parenchyma. Focal autoimmune pancreatitis at the pancreatic head is often characterized by the sole dilation of the common bile duct^[8]. The vascularization of autoimmune pancreatitis can be demonstrated by CEUS, which shows most often a moderate or marked^[8] enhancement in the early contrast-enhanced phase, though inhomogeneous due to the thinning of the glandular vessels as the consequence of the thick lymphocytic infiltration and fibrosis. In our study, parenchymographic enhancement, defined as slight continuous enhancement inside the pancreatic masses with isovascularity to the adjacent pancreatic parenchyma, was shown in 94% of the mass-forming pancreatitis. These CEUS findings have been reported to be especially useful in the study of focal forms of autoimmune chronic pancreatitis, in which differential diagnosis with ductal carcinoma is a priority^[11]. The ability of EUS as well other imaging modalities to differentiate pancreatic cancer from pseudotumorous chronic pancreatitis is reported in literature^[18]. Our data suggests that contrast-enhanced ultrasonography should be used in the characterization of pancreatic masses as complementary to CT and MRI. The sensitivity of CEUS in the identification of inflammatory masses allows to propose to obtain a diagnosis with fine needle percutaneous cytology in pancreatic focal masses that show glandular parenchymography at the examination. However, our study is surely limited by the retrospective evaluation.

In conclusion, CEUS allows diagnosis of mass-forming pancreatitis with diagnostic accuracy of 96%. CEUS significantly increases the diagnostic confidence in respect to basal US in discerning mass-forming pancreatitis from pancreatic neoplasm.

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Predictive factors of survival in patients treated with definitive chemoradiotherapy for squamous cell esophageal carcinoma

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Abstract

AIM: The aim of the study was to evaluate the predictive factors of survival in patients with locally advanced squamous cell esophageal carcinoma (LASCOC) treated with definitive chemoradiotherapy (CRT) regimen based on the 5FU/CDDP combination.

METHODS: All patients with LASCOC treated with a definitive CRT using the 5FU/CDDP combination between 1994 and 2000 were retrospectively included. Clinical complete response (CCR) to CRT was assessed by esophageal endoscopy and CT-scan 2 mo after CRT completion. Prognostic factors of survival were assessed using univariate and multivariate analysis by the Cox regression model.

RESULTS: A total of 116 patients were included in the study. A CCR to CRT was observed in 86/116 (74.1%). The median survival was 20 mo (range 2-114) and the 5-year survival was 9.4%. Median survival of responder patients to CRT was 25 mo (range 3-114) as compared to 9 mo (range 2-81) in non-responder patients ($P < 0.001$). In univariate analysis, survival was associated with CCR ($P < 0.001$), WHO performance status < 2 ($P = 0.01$), tumour length < 6 cm ($P = 0.045$) and weight loss $< 10\%$ was in limit of significance ($P = 0.053$). In multivariate analysis, survival was dependant to CCR ($P < 0.0001$), weight loss $< 10\%$ ($P = 0.034$) and WHO performance < 2 ($P = 0.046$).

CONCLUSION: Our results suggest that survival in patients with LASCOC treated with definitive CRT was correlated to CCR, weight loss and WHO performance status.

INTRODUCTION

Esophageal cancer is a frequent gastrointestinal malignancy with 32 332 new cases per year in Europe. In France, esophageal carcinoma is the third most frequent digestive tract cancer with approximately 5000 new cases per year^[1,2]. Approximately 50% of patients present a locally advanced esophageal carcinoma at diagnosis. To date, the incidence rate of adenocarcinoma is increasing but squamous cell carcinoma still remains the most frequent histological type in France^[1,2]. The definitive chemoradiotherapy (CRT) based on the Herskovic regimen is considered as the standard medical treatment in non operated patients with locally advanced esophageal carcinoma^[3,4]. Moreover, two phase III trials recently suggested that definitive CRT could be considered as an alternative treatment in patients with esophageal carcinoma^[5,6].

However, some questions remain unsolved as regards the CRT regimen optimisation. Moreover, although most studies included both patients with squamous cell and adenocarcinoma, it has been suggested to consider these two tumours separately for treatment as regards their different risk factors, carcinogenesis pathways and treatment response^[3-5,7-11]. Furthermore, the study of Rizk *et al* recently reported that long term prognosis in patients with esophageal carcinoma treated with preoperative CRT could be significantly different according to the histological type of tumour^[11]. However, few reported series have specifically focused on the long-term survival analysis in patients with squamous cell carcinoma treated with definitive CRT using the 5FU/CDDP combination^[12-20]. Moreover, most of these series included a limited number of patients or reported results with short follow-up^[13-20].

The aim of the present study was to assess the long-

term results and the predictive factors of survival in a large series of patients with a locally advanced squamous cell esophageal carcinoma (LASCOC) treated with a definitive CRT regimen based on the 5FU/CDDP chemotherapy (CT) combination. Furthermore, knowledge of these prognostic factors could be useful for the management of individual patients as well as a stratification variable for the design of future randomised trials.

MATERIALS AND METHODS

Patient population

All consecutive patients with a LASCOC referred between January 1994 and the 31st December 2000 were retrospectively included for the study. Patients were selected based on the following criteria: a histologically confirmed squamous cell carcinoma; a first-line treatment with a definitive CRT regimen using the 5FU/CDDP CT and concomitant external radiotherapy (RT). Patients were excluded if they had previous a history of carcinoma during the past three years and if they had synchronous distant metastasis. In our centres, the definitive CRT regimen based on the 5FU/CDDP combination was the first therapeutic option used in patients with LASCOC.

For each patient, we routinely recorded all baseline clinical and tumour characteristics including age, sex, World Health Organisation (WHO) performance status, dysphagia Atkinson score and weight loss at the beginning of treatment, median tumour length, esophageal tumour location, and tumour stage. Events and toxicity related to treatment were also included in the computer data base.

Tumour stage

The 1983 AJCC staging system was used in this study according to recently published recommendations^[21]. Tumour evaluation was based on oesophagoscopy, barium oesophagography, chest and abdominal computed tomography (CT-scan), endoscopic bronchoscopy and esophageal ultrasonography when feasible.

Treatment schedule

CRT regimen was based on the 5 FU/CDDP CT combination associated with an external RT. The RT was delivered either by a dose of 50 Gy (50 Gy/25 fractions per 5 wk) with concomitant CT courses delivered on wk 1 and 5, or either a dose of 60 Gy (20 Gy/10 fractions × 3 courses separated by a 2-wk break) with concomitant CT courses delivered on wk 1, 5 and 9. The CT courses combined 5-FU (750 to 1000 mg/m² per day delivered by continuous infusion on 4 d) and CDDP (75 to 100 mg/m² delivered on 1 d). The target volume of RT was the macroscopic tumour and enlarged lymph nodes, if any, surrounded by 5 cm proximal and distal margins and a 2 cm radial margin. The target was extended to the inferior cervical area in cases of tumours located above the carina. The specified dose was delivered at the intersection of the central axis of the beams, according to international guidelines. The irradiation technique was applied in anterior and posterior opposed fields. At 40 Gy, the radiation portals were reduced to shield the spinal cord and encompass the primary tumour with a 2-3 cm craniocaudal

margin.

Evaluation of clinical response and toxicity to CRT

Patients were considered to have a clinical complete response (CCR) to CRT when no residual tumour was identified on endoscopy and when no metastatic disease occurrence was observed on CT-scan. This evaluation was performed 2 mo after CRT completion.

Toxicity related to the treatment was evaluated using the National Cancer Institute Common Toxicity Criteria (NCI-CTC, version 2.0). Toxicity was assessed in each patient at d 1 of each chemotherapy course. At each course, patients received the treatment when they exhibited a WHO performance status of 2 or less; satisfactory haematologic function (leucocytes count $\geq 3000 \text{ mm}^{-3}$, platelet count $\geq 100000 \text{ mm}^{-3}$) and good renal function (creatinine serum level $\leq 100 \text{ micromole/L}$). Patients with major complication i.e. heart disease, pulmonary fibrosis, or active carcinoma at the other site were not eligible for treatment.

Follow-up

The follow-up was performed on clinical basis, endoscopy and CT scan. Histopathological confirmation of the recurrence was not routinely required. Follow-up was performed either until death or for the purpose of this study until October 2005.

Statistical analysis

Analysis was performed in October 2005 and was considered the cut-off date. Survival curves according to the putative prognostic factors were established using the Kaplan-Meier method and were compared with a log-rank test. The effects of clinical characteristics at baseline related to prognosis using univariate analysis were further evaluated in multivariate analysis using Cox regression model. A two-side *P*-value equal or less than 0.05 was considered to indicate statistical significance. Data from patients who had been lost to follow-up were censored at the time of last obtained information. The date of CRT initiation was the starting point for the analysis of overall survival. The date of CRT response evaluation was the starting point for the analysis of the disease free survival.

RESULTS

Patients characteristics

Between the first of January 1994 and the 31st December 2000, one hundred and sixteen consecutive patients were treated with a CRT based on the 5FU/CDDP CT combination. The majority of patients had a good performance status and the dysphagia score prior to CRT reflected their ability to eat a normal or semisolids diet for approximately 90% of these patients (Table 1). Among patients who were estimated with T1-T2 tumour on CT-scan, 14 were estimated to present with a T1-T2 N0 tumour. These latter patients were treated with a definitive CRT as regards age and/or comorbidities.

Safety and toxicity per patient

Significant toxicities per patient are shown in Table 2.

Table 1 Patient characteristics

	n = 116	%
Mean age (yr)	61.3 (40-90)	
Male	101	87.1
Female	15	12.9
WHO performance status (OMS)		
0	32	27.6
1	70	60.3
2	14	12.1
Dysphagia (Atkinson score)		
0	2	1.7
1	21	18.1
2	58	50
3	26	22.4
4	9	7.8
Weight loss \geq 10% at CRT		
Initiation	30	25.9
TNM		
T 1-2	27	23.3
3-4	89	76.7
N 0	50	43.1
1	54	46.5
x	12	10.4
M 0	116	100
1	-	-
Esophageal location		
Upper one-third	35	30.2
Middle one-third	53	45.7
Lower one third	28	24.1
Mean tumour length (cm)	4.9 (0-15)	
Histopathology		
Squamous cell carcinoma	116	

Table 2 Significant treatment toxicities per patient (%)

	Grade 3 (%)	Grade 4 (%)
Haematological		
Neutropenia	9	3
Anemia	11	-
Thrombopenia	1	1
Mucositis		
Oral	4	-
Esophageal	16	-
Gastrointestinal		
Nausea	12	-
Diarrhoea	4	-
Neuropathy	1	-
Alopecia	3	-

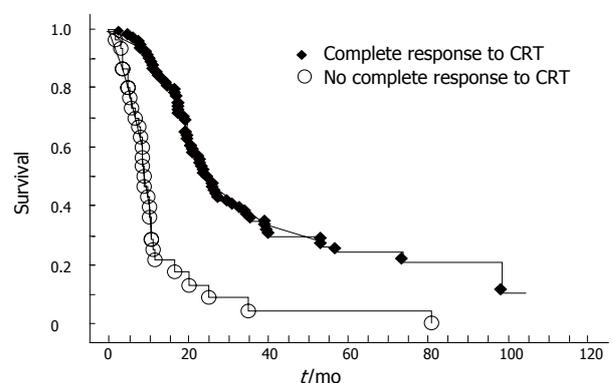


Figure 1 Survival according to response to CRT. The median overall survival of patients who had a complete clinical response (CCR) to the chemoradiotherapy (CRT) was 25 mo as compared to 9 mo in non-responder patients ($P < 0.001$).

There was no death related to CRT. Moreover, 75 patients (64.6%) experienced grade 3-4 toxicities and 112 (96.5%) patients achieved the planned concomitant CRT regimen. Dose modification to the planned CT regimen were required in 55 patients (47.4%) and 18 patients (15.5%) received at least one of their CT courses with a delay of more than 1 wk. The mean delivered radiation dose was 53.2 Gy.

During the CRT treatment, 18 patients (15.5%) required nutritional enteral feeding. In contrast, palliation of the dysphagia by endoscopic stenosis dilation was performed in 25 patients (21.6%). A self expandable metallic stent was inserted in 6 patients (5.1%) during CRT treatment.

Clinical complete response (CCR) to CRT

A total of 86/116 patients (74.1%) achieved a CCR to CRT. In the remaining 30 non-responder patients, a self expanding metallic stent was inserted in 12 for dysphagia palliation after completion of CRT, a CT treatment was initiated in 1 patient and salvage surgery was performed in 2 patients.

Patient outcome

In October 2005, 7/116 patients (6%) were still alive. The median follow-up of these surviving patients was 79 mo (range 56-104) and the median follow-up of the 11

patients who were lost to follow-up during the study was 45.1 mo (range 9-68).

The median overall survival was 20 mo (range 2-114) and the 2-years and 5-years survival rates were 39.6% and 9.4%, respectively. Moreover, the median overall survival of the 86 patients who had CCR to CRT was 25 mo (range 3-114) as compared to 9 mo (range 2-81) in non-responder patients ($P < 0.001$) (Figure 1). The median disease free survival of responder patients to CRT was 17 mo.

During the follow-up, 34 of responder patients (39.5%) experienced a local disease recurrence, 37 patients (43%) experienced metastatic disease and 19 of them experienced both of these recurrences.

Prognostic factors of survival

As regards univariate analysis (Table 3), survival was correlated to CCR to CRT ($P < 0.001$), WHO performance status < 2 ($P = 0.01$) and tumour length < 6 cm ($P = 0.045$). In contrast, weight loss $> 10\%$ at the start of CRT was in limit of statistical significance and was included in multivariate analysis ($P = 0.053$). In a Cox regression model (Table 3), the independent covariates significantly associated with survival were the CCR to CRT ($P < 0.0001$; Odds Ratio (OR): 0.121; IC95 = 0.06-0.24), the weight loss $< 10\%$ ($P = 0.034$; OR: 0.53; IC95 = 0.29-0.95) and a WHO performance status < 2 ($P = 0.046$; OR: 0.495; IC95 = 0.24-0.99).

DISCUSSION

To date, definitive CRT based on the 5FU/CDDP combination is considered as standard treatment in non operable patients with locally advanced esophageal carcinoma whatever the histological tumour phenotype^[3-5]. Some authors recently suggested that histological types of oesophagus tumour could be considered separately regarding their significant different treatment response and long term prognosis^[9-11]. Therefore, we performed a retrospective analysis of the long term outcome and predictive factors of survival in 116 patients with LASCOC treated with a definitive CRT using the 5FU/CDDP CT combination.

In our study, the 5-year survival was 9.4% and the median overall survival was 20 mo. We also found that responder patients to CRT had a significantly increased median survival as compared to non-responders patients (24 mo *vs* 9 mo; $P < 0.001$). This result was supported by the multivariate analysis which identified the CCR as an independent prognostic factor of survival. In definitive CRT series using the 5FU/CDDP combination, a survey of literature showed that median overall survival ranged from 17 to 26 mo and the 5-year survival rate from 20% to 30%^[12-20]. The 5-year survival rate in our study was slightly lower as compared to those reported in these series. This result could be explained by the patient selection bias in these prospective trials, whereas our retrospective study possibly reflected the outcome of non-selected patients with LASCOC treated with definitive RT.

The CCR to CRT was obtained in 75.9% of patients in our series. Moreover, a CCR was identified as an independent prognostic factor of long-term survival in our multivariate analysis. Although the prognostic significance of pathological complete response after preoperative CRT was well documented^[22-24], to our knowledge, there are no previous studies that have reported similar result in patients with LASCOC treated with definitive CRT using the 5FU/CDDP combination. In fact, the significant impact of CCR to CRT in long-term survival in patients treated with the same definitive CRT regimen was reported in series which included patients with mixed histological tumour types^[12,25,26]. Moreover, in the reported Ohtsu *et al* study focusing exclusively on patients with LASCOC, the CCR to CRT was identified as a predictive factor of the progression free survival but not for overall survival^[18]. In our study, 39.5% of responder patients to CRT had a local disease recurrence. In previous studies, local recurrences were reported to be as high as 38% to 48% after definitive CRT^[12,18]. Furthermore, a distant metastasis occurred in 43% of responder patients to CRT in our series. This result compared less favourably to other series including patients with squamous cell carcinoma treated with the same CT combination, where less than 30% of responder patients to CRT experienced a distant metastasis^[12,18]. The frequent use of additional CT in responder patients to CRT in these latter series could probably explain the difference in metastasis frequency. Indeed, only 44% of patients received additional CT after the CRT completion in our study.

Thus, our results suggest that further optimisations of

Table 3 Predictive factors of survival, univariate and multivariate analysis

	Univariate		Multivariate	
	P	P	OR	IC95
Sex	0.507	-		
Age < 70	0.745	-		
WHO performance status < 2	0.01	0.046	0.52	0.28-0.99
Weight loss < 10%	0.053	0.034	0.53	0.29-0.95
Dysphagia	0.074	-		
T	0.273	-		
N	0.499	-		
Tumour location	0.501	-		
Tumour length < 6 cm	0.045	0.534	0.86	0.53-1.38
Complete response to CRT	< 0.001	< 0.000	0.21	0.13-0.36

the definitive CRT regimen are required for both local and systemic disease control improvement. To date, the 5FU/CDDP combination used in our study is still considered the standard for the CRT regimen. Evaluation of novel chemotherapy regimens which include new drugs such as irinotecan and new cancer therapies encompassing those directed against vascular growth factor and epidermal growth factor pathways may be usefully associated with RT to provide improved sustained CCR and therefore optimal long-term survival^[27-30]. Furthermore, CRT regimen optimisation should determine the optimal radiotherapy dose in order to achieve a sustained local disease control. In our study, patients received a mean dose of 52.1 Gy of external RT in the tumour bed, which was a similar dose as that used in the standard regimen described by Herskovic *et al*^[31]. Recently, Zhang *et al* reported, in a retrospective study, that patient who received a dose of RT more than 51 Gy had a statistically better local disease-free survival and overall survival as compared to patients treated with a dose of RT less than 51 Gy^[31]. Minsky *et al* specifically investigated dose escalation in RT. In this prospective study, patients were randomised in a CRT regimen using either 50.4 Gy of RT or 64.8 Gy of RT^[32]. However, the unexplained excess of death frequency (9%) which occurred prior to the dose escalation in patients treated in the 64.8 Gy arm did not permit a valid conclusion as regards the optimal dose of RT. In our study, no death due to CRT was observed. However, we found that 64.6% of patient experienced grade 3-4 toxicities, which was a similar result to that usually reported^[3,4]. As regards the new RT techniques including three dimensional CT planning with conformal beam, dose escalation in the definitive CRT regimen could be reconsidered. Further studies including these new RT techniques may be helpful to evaluate the optimal and safety dose of RT in patients with LASCOC.

Multivariate analysis identified the WHO performance status less than 2 as the second independent predictive factor of long-term survival in patients treated with definitive CRT for a LASCOC. The WHO performance status has been previously identified as prognostic factor in patients with esophageal cancer treated by radiotherapy alone or in patients with metastatic oesophago-gastric cancer in previous studies^[21,33]. Moreover, Polee *et al* also identified this variable as a prognostic factor of survival in

a meta-analysis performed in six prospective trials which included several CRT regimens and mixed histological type of esophageal tumour^[34]. However, to our knowledge, this result had never been previously reported in patients with LASCOC treated in curative intent with definitive CRT using the 5FU/CDDP combination. The WHO performance status evaluation provides useful information on the patient's general well-being indirectly reflecting the impact of the malignancy on the physiological individual condition. Although the evaluation of the WHO performance status seems to be subjective, our results showed that this variable was significantly correlated with survival. Moreover, weight loss before treatment starting was also identified as prognostic factor in our work. This variable was recently to be correlated with prognosis in a meta-analysis by Thomas *et al* in patient treated by definitive CRT for esophageal cancer^[30].

Interestingly, tumour characteristics were not identified as prognostic factors in our study. Indeed, the tumour diameter and the tumour length were only significant in univariate analysis. In surgical series, the extent of the tumour infiltration in the esophageal wall and the lymph node involvement were frequently closely correlated with survival^[23,24,35]. Although an underpowered significance could not be excluded in the analysis, our results could also reflect the limited accuracy of the usual staging evaluation including oesophagography, oesophagoscopy and thoraco-abdominal computed tomography used during the period of the study. However, these imaging modalities appear to be more accurate for the CRT response assessment as regards the significant link that was observed between the CCR and survival. The high rate of early disease recurrence in our study could reflect an overestimation of the CCR rate by modalities used in our study. Thus, the further use of endoscopic ultrasound and PET-FDG, may be helpful to provide better correlative data for initial tumour staging and for tumour response assessment^[36-38].

In conclusion, based on a large series of patients with LASCOC, our results suggest that survival of these patients treated with definitive CRT using the 5FU/performance status. These prognostic factors could be considered for the management of individual patients as well as a stratification variable for the design and interpretation of further randomised trials. However, as regards the retrospective design of our study, further prospective studies are necessary to investigate the impact of these prognostic factors.

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Pegylated-interferon alpha 2a treatment for chronic hepatitis C in patients on chronic haemodialysis

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Abstract

AIM: To evaluate the response to pegylated-interferon alpha 2a in chronic hepatitis C patients on chronic haemodialysis.

METHODS: Ten patients with chronic C hepatitis were enrolled in this study. All had increased aminotransferases for more than 6 mo, positive antiHCV antibodies and positive PCR HCV-RNA. We administered Peg-Interferon alpha 2a 180 µg/wk for 48 wk. After 12 wk of treatment we evaluated the biochemical and early virological response (EVR). At the end of the treatment we evaluated the biochemical response and 24 wk after the end of the treatment we evaluated the sustained virological response (SVR). We monitored the side-effects during the treatment.

RESULTS: Two patients dropped out in the first 12 wk of treatment and 2 after the first 12 wk of treatment. After 12 wk of treatment, 7 out of 8 patients had biochemical response and EVR and 1 had biochemical response but persistent viremia. We had to reduce the dose of pegylated-interferon to 135 µg/wk in 2 cases. Three out of 6 (50%) patients had SVR 24 wk after the end of the treatment. Intention-to-treat analysis showed that 3 out of 10 patients (30%) had SVR. Side-effects occurred in most of the patients (flu-like syndrome, thrombocytopenia or leucopenia), but they did not impose the discontinuation of treatment.

CONCLUSION: After 12 wk of treatment with Peg-Interferon alpha 2a (40 ku) in patients on chronic haemodialysis with chronic C hepatitis, EVR was obtained in 87.5% (7/8) of the cases. SVR was achieved in 50% of the cases (3/6 patients) that finished the 48 wk of treatment.

INTRODUCTION

Although constant efforts have been made to improve the outcome of hepatitis C patients, chronic infection with hepatitis C virus (HCV) remains a problem for hepatologists. The development of new therapeutic formulas (pegylation) and the introduction of ribavirin were major steps forward. However the problem is not entirely solved since the sustained virological response can be obtained in only half of HCV-infected patients. There are also the special groups of patients (with liver cirrhosis, with HIV coinfection, patients on chronic haemodialysis) in which the optimal antiviral treatment is still not established.

In patients on chronic haemodialysis, the number of individuals infected with HCV is rather high mostly due to nosocomial infection. The reported prevalence of HCV infection ranges from 8% to 20% in dialysis patients in developed countries^[1-5] and much higher in less developed countries^[6]. The prevalence of anti-HCV among dialysis patients was 43.9% in Saudi Arabia in 2001^[7], 30% in India in 2002^[8], and 41% in Turkey (2001)^[9]. In United States of America in 2000, 8.4% of haemodialysis patients were anti-HCV positive^[5]. The incidence of HCV infection is higher in patients undergoing dialysis at hospitals than in those undergoing haemodialysis or peritoneal dialysis at home.

The main mechanisms involved in nosocomial infection with HCV in haemodialysis patients are filter reuse, use of contaminated haemodialysis machines and contamination of medical staff's hands^[10]. It has been proven that the incidence of HCV infection in haemodialysis patients increases if the nurse does not change her gloves before injecting each patient^[11] and if HCV (+) patients undergo haemodialysis in the same room with HCV (-) patients^[12].

Other possible risk factors for transmission of the virus are sharing single vials to prepare drugs or infusions for different patients, distance less than one meter between chairs^[13], sharing a single heparin-saline solution ampoule in different patients^[14]. A large French multi-center study^[15] on 1323 haemodialyzed patients has shown an incidence of 0.4% new HCV infections per year, almost two thirds of them occurring in infected patients on dialysis during the same shift at the same unit.

Regardless of the route of infection, the evolution of HCV-infected patients on chronic haemodialysis is often severe. Martin *et al*^[16] showed that 24% of haemodialysis patients with positive anti-HCV Ab have liver cirrhosis and that there is no correlation between the severity of hepatic lesions and viral genotype, viral load or transaminase level. Hence we must treat chronic hepatitis C in haemodialysis patients, particularly in those on the waiting list for renal transplantation, because post-transplant immunosuppressive therapy can accelerate the natural course of the liver disease. Interferon-based therapy is not recommended in HCV positive patients after renal transplantation due to a significant risk of graft loss and a low rate of clearance of the virus^[17,18]. Also ribavirin monotherapy for renal transplant recipients positive for anti-HCV is associated with improvement in liver enzymes but not significant change of HCV RNA^[19]. On the other hand, Kamar *et al*^[20] showed that treatment of HCV positive haemodialysis patients with interferon α could induce complete and sustained clearance of the virus in almost 29% of them, without any relapses after renal transplantation despite subsequent immunosuppressive treatment.

Another problem of the treatment for HCV-infected patients on dialysis is the contraindication of ribavirin, due to the risk of deep and long-lasting haemolytic anaemia^[21].

Due to these characteristics of this special group of patients and the promising results of our previous study using standard interferon in haemodialysis patients^[22], we decided to evaluate the effect of pegylated-interferon in patients with chronic hepatitis C on dialysis.

MATERIALS AND METHODS

We included 10 haemodialysis patients in our study (4 males and 6 females, mean age 40.2 years). Written informed consent to participate in this study was obtained from all of them. All had increased aminotransferases for more than 6 mo, anti-HCV antibodies (Elisa III) and positive PCR HCV-RNA. The viral load at admission and after 12 wk of treatment (EVR) was determined by the classical polymerase chain reaction (Roche) with a detection limit of 600 UI/mL. The viral load 24 wk after the end of treatment (SVR) was determined by real-time PCR (Abbott) with a detection limit of 23 UI/mL. None of the patients presented with clinical, biological, endoscopic or ultrasound signs of liver cirrhosis. We did not perform liver biopsy because of the increased risk of bleeding in haemodialysis patients.

All patients were treated with pegylated-interferon alpha 2a (180 μ g/wk) for 48 wk. We evaluated the biochemical response every month and the virological response after 12

wk of treatment (early virological response-EVR) and 24 wk after the end of treatment.

We monitored the side effects during the treatment. At the end of the treatment we evaluated the biochemical response of our patients (number of patients with normal transaminases) and the sustained virological response (SVR) 24 wk after the end of the treatment (72 wk from the beginning of the treatment) by determining the virological load.

RESULTS

The 10 patients studied are listed in Table 1. At the beginning of the study the virological load was low in 2 patients (< 10 kIU/mL), moderate in 5 patients (10-500 kIU/mL), and high in 3 patients (> 500 kIU/mL). Two patients were excluded from the study. One patient was excluded because of lack of compliance and 1 patient discontinued the treatment due to complications after surgery (sepsis).

We determined the biochemical and virological response (PCR RNA HCV) in the 8 patients who continued the treatment after 12 wk of therapy. Of these patients, 7 (87.5%) had biochemical response (normal transaminases) as well as virological response (viral load < 0.6 kIU/mL), 1 (12.5%) had biochemical response (normal transaminases) but persistent viremia. We continued the treatment with pegylated-interferon alpha 2a for 48 wk. During this period one patient died of cerebral haemorrhage caused by arterial hypertension after 16 wk of therapy (the patient having normal prothrombin time and only mild thrombocytopenia-104000 platelets/mL) and one patient was excluded from the study due to lack of compliance after 28 wk of therapy.

The total number of patients who finished the 48-wk treatment was 6 (60%). All of them had biochemical response at the end of treatment (normal transaminases). Three out of 6 patients (50%) had sustained virological response (SVR) 24 wk after the end of the treatment. The intention-to-treat analysis showed that 3 out of 10 patients (30%) had sustained virological response 24 wk after the end of the treatment.

All patients had minor flue-like symptoms, 4 had mild thrombocytopenia ($Tr < 150\,000/mm^3$) and 2 had moderate thrombocytopenia ($Tr < 100\,000/mm^3$), 4 had transitory mild leucopenia ($L < 4000/mm^3$). In the 6th mo of therapy one of the patients developed sepsis secondary to central venous catheter infection. During this period the patient had elevated transaminases. Unfortunately, this patient abandoned the treatment one month later.

We modified the dose of pegylated-interferon in 2 patients. In one we reduced it to 135 μ g/wk for 1 mo (because of the thrombocytopenia and haemorrhagic complications-metroragia, epistaxis, prolonged bleeding of the fistula), then 180 μ g/wk was administered again. In the second patient the dose reduction to 135 μ g/wk was initiated in the 4th mo of therapy until the end of 48-wk treatment.

We did not stop the treatment in any patient due to severe side effects of pegylated-interferon.

Table 1 Demographic data of haemodialysis patients treated with pegylated interferon

No.	Patient	Sex	Age	Duration of treatment wk	Stop of because treatment of	Initial viral load (UI/mL)	Viral load at wk 12 (UI/mL)	Viral load at wk 72 (UI/mL)
1	B.L.	F	42	48		4090	< 600	< 23
2	C.A.	M	34	48		84 760	< 600	45
3	B.V.	M	45	48		252 000	< 600	263 300
4	L.D.	F	40	6	Non compliance	8000	-	-
5	B.L.	F	44	48		157 000	< 600	< 23
6	P.E.	F	28	48		+	< 600	< 23
7	C.M.	F	47	48		766 000	60 100	178
8	C.A.	F	45	8	Complication after surgery	417 000	-	-
9	T.M.	M	26	16	Death of cerebral haemorrhage	> 1 000 000	< 600	-
10	P.C.	M	51	28	Non compliance	> 1 000 000	< 600	-

DISCUSSION

Many clinical trials have focused on the treatment of chronic hepatitis C patients on chronic haemodialysis with standard interferon, because ribavirin is not recommended. Some studies have used ribavirin at low doses (170-300 mg/d) together with standard interferon^[23]. The results are encouraging but a careful monitoring of anaemia is mandatory. When anaemia occurs it is corrected with high doses of erythropoetin. On the other hand, post-transplant treatment of chronic C hepatitis with interferon is not recommended because it can induce graft rejection (15.4%-63.6% of cases)^[24]. Also, post-transplant monotherapy with ribavirin or amantadine has been proven inefficient^[24].

Fabrizi *et al*^[6] have found a mean SVR of 37% and a mean dropout rate of 17% in chronic hepatitis C patients on dialysis after interferon monotherapy. Our experience in treatment of these patients with standard interferon showed that sustained biochemical response is 46.1% and sustained virological response (HCV-RNA) is 38.4% respectively 6 mo after interferon treatment^[22].

The promising results of monotherapy with standard interferon in chronic haemodialysis patients with chronic hepatitis C^[22,25-27], have shown that viral clearance occurs in 27%^[27] to 64%^[26] of patients after 12 mo of treatment with standard interferon.

In patients on chronic haemodialysis, the combined treatment with interferon and ribavirin is difficult to manage because haemolysis is induced by ribavirin. There are studies in which ribavirin is administrated at low doses (170-300 mg/d), the results are remarkable but anaemia should be carefully monitored^[23].

The second therapeutic option for patients on chronic haemodialysis with chronic C hepatitis is to use pegylated interferon. In most of the studies performed in patients with chronic C hepatitis and normal renal function, the response rate doubled when the patients switched from standard interferon to pegylated-interferon. Some 3rd phase studies have been performed in Greece, Mexico, Great Britain and USA to evaluate the sustained virological response after treatment with pegylated interferon alpha 2a in patients on chronic haemodialysis.

Martin *et al*^[28] demonstrated that the absorption, distribution and total clearance of pegylated-interferon alpha 2a (40 ku) are not very different from those in

patients with normal renal function, and that the tolerability of pegylated-interferon alpha 2a and the adverse effects in patients on chronic haemodialysis are similar with those in patients without renal impairment. In our group the side effects were quite the same with those in "normal" patients with chronic hepatitis C treated with pegylated-interferon.

We reduced the dose of pegylated-interferon to 135 µg/wk in 2 cases (in one patient only for one month and in another until the end point of treatment). Various authors have recommended a dose of 180 or 135 µg/wk of pegylated-interferon alpha 2a in patients on chronic haemodialysis. We prefer to start with 180 µg/wk in order to reduce the dosage if severe side effects occur. We reduced the dosage in 2 patients due to thrombocytopenia and bleeding.

Data on the patients on haemodialysis treated with pegylated-interferon alpha 2b are rather discouraging. A study by Russo *et al*^[29] on the HCV-infected patients on haemodialysis treated with pegylated-interferon alpha-2b showed that a poor tolerance is associated with substantial side effects. Also, a case report by Potthoff *et al*^[30] showed that IFN-alpha 2b three times a week after haemodialysis seems to be better tolerated than pegylated-interferon-alpha 2b once a week. A randomized study performed by Mahmoud *et al*^[31] in pretransplant haemodialysis patients with chronic hepatitis C treated with standard interferon alpha 2b, showed that IFN-treated patients have significantly better post-transplant hepatic functions and significantly lower rates of chronic allograft nephropathy. Further studies are needed to find out which type of interferon is better tolerated and has better results for the treatment of haemodialyzed patients with chronic hepatitis C.

Since there are more and more encouraging results of treatment with interferon in patients on chronic haemodialysis with chronic hepatitis C, it is likely that very soon all these patients can benefit from antiviral therapy (standard interferon alone or in combination with ribavirin, or pegylated-interferon).

After 12 wk of treatment with Peg-Interferon alpha 2a (40 ku) in patients on chronic haemodialysis with chronic C hepatitis, the early virological response (EVR) (HCV-RNA absent by PCR) was obtained in 87.5% (7/8) of the cases. All the patients that finished the 48 wk of treatment had normal transaminases (biochemical response) (6/6). We had to reduce the dose of Peg-Interferon in only 2 cases. Even if side effects occurred in most of the patients

(flue-like syndrome, thrombocytopenia or leucopenia) they did not impose the discontinuation of treatment. The sustained virological response at 6 mo after the end of the therapy was achieved in 50% of the cases (3/6 patients) that finished the course of 48 wk of treatment.

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Circulating hTERT mRNA as a tumor marker in cholangiocarcinoma patients

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diagnosis of cholangiocarcinoma.

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Abstract

AIM: To investigate human telomerase reverse transcriptase (hTERT) mRNA in the serum of cholangiocarcinoma patients.

METHODS: The serum of thirty three cholangiocarcinoma patients, forty one benign biliary tract disease patients and ten healthy volunteers were collected and analyzed for the expression of hTERT mRNA by real-time reverse transcriptase-polymerase chain reaction (RT-PCR). We then examined the correlation between values of serum hTERT mRNA and the pathological staging of cholangiocarcinoma.

RESULTS: hTERT mRNA was detected in 28 of 33 (84.85%) of serum obtained from cholangiocarcinoma patients and 9 of 41 (21.9%) of serum obtained from benign biliary tract disease patients. hTERT mRNA was not detected in any serum obtained from healthy volunteers. on the other hand the common tumor marker, CA19-9 was detected in 20 of 33 (60.6%) of serum obtained from cholangiocarcinoma patients and 8 of 41 (19.5%) of serum obtained from benign biliary tract disease patients. However, no correlation was found between the present of serum hTERT mRNA and tumor staging.

CONCLUSION: These results indicate that the detection of circulating hTERT mRNA was identified in almost all cholangiocarcinoma patients. It offers a novel tumor marker, which can be used as a complementary study for

INTRODUCTION

Cholangiocarcinoma is the cancer arising from cholangiocyte, the epithelial cells lining the intrahepatic and extrahepatic bile ducts. It is one of the most common liver cancers in the population of Northeast Thailand and responsible for approximately one in five cancer-related deaths among Thai patients^[1]. Three-year survival rates of 40%-60% have been reported only in a few number of patients resected for cure^[2]. Diagnosis of cholangiocarcinoma is often difficult. It requires multiple complementary studies including evaluation of clinical symptoms, imaging, and tumor markers. Tissue biopsy and cytology have poor sensitivity and are positive only in about 30% of cases of cholangiocarcinoma. Recently, the percentages of positive serum obtained from the common marker (CA19-9) are only less than 70%^[3]. In addition, CA19-9 can be elevated in cholestasis in the absence of malignancy, and following liver injury. Thus, their accuracy for the diagnosis of cholangiocarcinoma is limited. It is necessary to find novel markers to use in diagnosis and treatment.

The human telomerase, which composed of two subunits including telomerase RNA template (hTR) and telomerase transcriptase protein (hTERT), functions as a reverse transcriptase enzyme in the process of telomere synthesis^[4]. Telomerase activity was detected in 85%-100% of cancer patients whereas normal somatic cells have low or undetectable^[4,5]. In addition, previous results demonstrated that circulating tumor-related RNA

including telomerase is frequently found in the plasma and serum of cancer patients^[6-8].

Consequently, telomerase activity is possibly used as a common molecular tumor marker in the serum. Previous studies also found a good correlation between the telomerase activity and the expression of hTERT subunit. The aim of this study is to test the usefulness of hTERT mRNA detection in the serum of cholangiocarcinoma patients by using real-time reverse transcriptase polymerase chain reaction.

MATERIALS AND METHODS

Cell lines

The human cholangiocarcinoma cell line HuCCA1 (kindly provided by Prof. Sirisinha, Department of Microbiology, Mahidol University) and RMCCA1 (established from Department of Surgery, Rajavithi Hospital) were grown in Ham's F12 medium supplemented with 100 mL/L fetal bovine serum at 37°C in a 5% (50 mL/L) CO₂ humidified atmosphere.

Patients and sample preparation

Thirty-nine informed and consenting patients undergoing surgery for cholangiocarcinoma at the Rajavithi Hospital, Thailand, between July 2003 and April 2006 were included in this study. Tumor samples were collected at the time of surgery and histopathologically characterized to confirm the diagnosis. Pathological data, including tumor staging was also collected. Fifty patients undergoing surgery for benign biliary tract disease were included in this study. Ten normal subjects were studied as negative controls.

Sample Collection

Blood was collected prior to surgery in plain tubes for serum sampling. After clotting, tubes were centrifuged at 1000 r/min for 15 min at room temperature, and serum was collected. This was followed by a second 15-min centrifugation at 1000 r/min to remove cellular debris. Serum samples were aliquoted and stored at -70°C until use. Serum CA19-9 level was measured in Clinical Laboratory of Rajavithi Hospital. The cut-off level was 100 IU/mL.

RNA Extraction

RNA from cell lines and serum was extracted using a commercially available kit (High Pure RNA Kit; Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's instructions. Only fresh or once-freeze thawed serum was used.

Real time RT-PCR for hTERT mRNA

Quantitative detection of hTERT mRNA was performed with the TeloTAGGG hTERT Quantification Kit (Roche Diagnostics GmbH, Mannheim, Germany), using the LightCycler system (Roche Diagnostics, Mannheim, Germany) for real-time PCR according to the manufacturer's instructions. For the reaction mixtures, 2 µL of hTERT reaction mix, 0.1 µL of reverse transcriptase, 2 µL of hTERT or PBGD mix, 13.9 µL of H₂O and 2 µL of standard RNA template or RNA from

Table 1 The patient demographic data and blood chemistry data

	Benign biliary tract disease	Cholangio-carcinoma	Healthy volunteers
Sex (Male:Female)	21:20	19:14	6:4
Age (year), (median)	53.68 (22-82)	56.40 (35-85)	49.50 (26-60)
SGOT (IU/dL)	105.32 ± 52.26	112.52 ± 44.54	30.20 ± 12.20
SGPT (IU/dL)	96.04 ± 45.42	73.41 ± 44.30	28.50 ± 9.22
Total Bilirubin (mg/dL) ^a	3.8 ± 2.21	12.8 ± 5.24	1.05 ± 0.25
Alkaline Phosphatase (U/dL) ^a	309 ± 67.53	550 ± 24.44	98 ± 10.60

^a*P* < 0.005.

serum samples was prepared. The reaction conditions were reverse transcription at 60°C for 10 min, followed by initial denaturation at 95°C for 30 s and 40 cycles of denaturation at 95°C for 0.5 s, annealing at 60°C for 10 s, and extension at 72°C for 10 s, respectively. The standard curve was established by determination of the five standards hTERT mRNA provided by the kit. The samples were normalized on the basis of the content of PBGD. Serum samples with more than 150 copies of PBGD suggesting an appropriate quality of RNA were used for the analysis of telomerase. Serum samples in which hTERT mRNA were detected were assigned to the hTERT-positive group.

Statistical analysis

Values were expressed as mean ± SD. Mean values were measured by the Student's *t* test. Correlations between serum hTERT mRNA and stage of cholangiocarcinoma were assessed using the chi-square test (χ^2). Sensitivity, specificity, positive predictive value, and negative predictive value were measured. *P* < 0.05 was considered as statistically significant.

RESULTS

Detection of hTERT mRNA in cholangiocarcinoma cells

The expression of hTERT mRNA in two cholangiocarcinoma cell lines (HuCCA1 and RMCCA1) was investigated. Quantitative real-time RT-PCR demonstrated definite expression of hTERT mRNA in both cholangiocarcinoma cell lines (Figure 1). This evidence confirmed the existence of telomerase activity in cholangiocarcinoma. Therefore, we attempted to detect the circulating hTERT mRNA in the serum of cholangiocarcinoma patients.

Detection of serum hTERT mRNA

Thirty-nine patients who were confirmed diagnosis as cholangiocarcinoma, fifty benign biliary tract disease patients and ten healthy volunteers were included in this study. Their serum was collected and extracted for total RNA. Only RNA samples, that could be detected by the expression of porphobilinogen deaminase (PBGD) as a housekeeping gene were included in this study. The thirty-three serum samples from cholangiocarcinoma patients, fortyone serum samples from benign biliary tract disease patients and ten serum samples from healthy volunteers detected for PBGD were as-

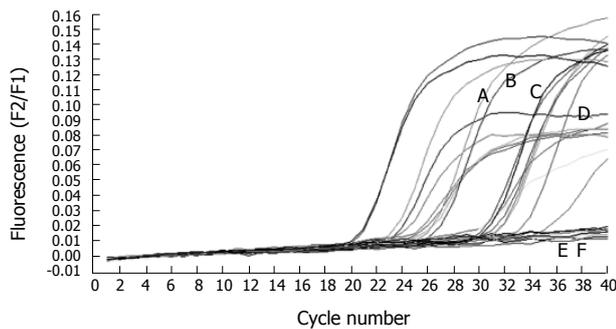


Figure 1 Continuously monitoring the development of signals in parallel standards and samples results in a series of amplification curves. The amplification of a 198 bp fragment of the generated hTERT cDNA was identified. (A: Signal from RMCCA1; B: Signal from HuCCA1; C, D: Signals from serum of cholangiocarcinoma patients; E, F: Signals from serum of benign biliary tract patients).

sayed for the expression of hTERT mRNA (Figure 1). The patients' demographic data was demonstrated in Table 1. There were no differences in sex, age, serum SGOT and serum SGPT between benign and cancer patients. However, total bilirubin and alkaline phosphatase were significantly high in cancer groups.

Serum hTERT mRNA was recognized in 28 of 33 cholangiocarcinoma patients (84.85%) and 9 of 41 benign biliary tract disease patients (21.9%). However, serum hTERT mRNA was not detected in any healthy volunteers. The efficiency of serum hTERT was compared with the serum CA19-9 as shown in Table 2. Serum hTERT was higher in sensitivity for detection of cholangiocarcinoma than serum CA19-9.

Detection of serum hTERT mRNA in relation to tumor stage

We also evaluated for the association between serum hTERT mRNA and the histopathological staging of cholangiocarcinoma in the surgical specimens resected from these patients. The result showed that serum hTERT mRNA could be detected in all stages of cholangiocarcinoma patients. However, it did not correlate with the staging of cholangiocarcinoma (Table 3).

DISCUSSION

Eukaryotic chromosomal ends consist of repeating DNA sequences (TTAAGG) termed telomeres. An enzyme that adds telomeric repeats onto chromosomal ends is telomerase. This enzyme is composed of two subunits; hTR and hTERT^[4]. Accordingly, both hTR and hTERT are necessary for telomerase activity, yet the catalytic activity of the enzyme is generally regulated through the presence and activity of hTERT. Therefore, the detection of hTERT mRNA is a guarantee for the present of telomerase activity^[8]. In most somatic cells in which telomerase activity is undetectable, telomeric sequences are lost with each cell division because of the end-replication problem. Unlike healthy cells, most malignant human cells are capable of escaping senescence and sustaining infinite proliferation through the activation of telomerase to

Table 2 Comparative analysis of serum hTERT mRNA and serum CA19-9 in diagnosis of cholangiocarcinoma

	Serum hTERT (%)	Serum CA19-9 (%)
Sensitivity	84.85	60.6
Specificity	78.05	80.49
Positive predictive value	75.68	71.43
Negative predictive value	86.49	71.74
False Negative	13.51	28.26
False Positive	24.32	28.6

Table 3 Detection of serum hTERT mRNA in relation to tumor staging

Tumor stages	Serum hTERT	mRNA
	positive	negative
Stage I	2	1
Stage II	17	3
Stage III	9	1

stabilize their telomere length^[4,8-12].

Traditionally, telomerase activity has been assessed based on a biochemical primer extension assay, the inefficiency and low sensitivity of which, together with the low amounts of telomerase activity, greatly limit the application of the assay in primary human tumors^[9]. Therefore, the detection of hTERT by real-time RT-PCR was used in this study. This assay determined the expression of telomerase by measuring the amount of the mRNA encoding its catalytic subunit hTERT. The relative telomerase expression levels were determined by comparing them to the expression levels of the housekeeping gene porphobilinogen deaminase (PBGD). hTERT-encoding mRNA from the serum was reverse transcribed and 198 bp fragments of the generated cDNA was amplified with specific primers in a one step RT-PCR reaction. The amplicon was detected by fluorescent emission using a specific pair of hybridization probes. In a separate RT-PCR, mRNA encoding for porphobilinogen deaminase (PBGD) was processed. The reaction product served as both a control for RT-PCR performance and as a reference for relative quantification.

Our study showed that hTERT mRNA was detected in almost all of the cholangiocarcinoma patients (84.85% of cases). However, in benign biliary tract disease patients, hTERT mRNA was also detectable (21.9% of cases). According to the previous report, telomerase activity has been reported in normal lymphocytes^[8-12]. This result suggested the contamination of lymphocytes in the serum specimens. Comparison with the common tumor marker, CA19-9 was detected in only 60.60% of cases. This data suggested that hTERT mRNA should be a candidate tumor marker in cancer patients.

hTERT mRNA is not specifically detected in serum of cholangiocarcinoma patients but it was also significantly found in several types of cancers such as breast cancer, malignant melanoma and thyroid cancer^[8]. Certainly, we have detected hTERT mRNA in the serum of five patients with hepatocellular carcinoma and three patients with pancreatic cancers (data not shown). This indicates that the

detection of circulating extracellular tumor-derived mRNA is not confined to any one cancer type, but may actually be a relatively ubiquitous finding across a broad range of cancers. However, no relationship was found between the expression of hTERT mRNA and clinicopathological findings. According with previous study, the presence of hTERT was unrelated to tumor size, tumor grade or the presence of nodal metastasis^[12]. These results suggest that the detection circulating hTERT mRNA does not predict prognosis in cholangiocarcinoma.

In conclusion, hTERT might serve as a tumor marker, which early identified circulating specific RNA originating from tumor cells. However, further examinations using more cholangiocarcinoma cases are necessary to evaluate the usefulness of this marker.

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Results of gastroscope bacterial decontamination by enzymatic detergent compared to chlorhexidine

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Abstract

AIM: To compare the efficacy of enzymatic detergent with chlorhexidine for gastroscope bacterial decontamination.

METHODS: A prospective randomized controlled study was undertaken to evaluate the ability of these 2 agents to achieve high level disinfection in a gastroscope. A total of 260 samples were collected from 5 different gastroscopes. Manual cleaning was done for 10 min with these 2 agents separately ($n = 130$ each). Then all specimens underwent 2% glutaraldehyde soaking for 20 min. After 70% alcohol was rinsed, sterile normal saline was flushed into each gastroscope channel and 40 mL of sample was collected. The sample was sent for aerobic bacterial culture after membrane was filtered. A colony count greater than 200 cfu/mL was considered significant.

RESULTS: The positive culture rate was 4.6% in the enzymatic detergent arm and 3.1% in the chlorhexidine arm. *Pseudomonas* species were the main organism detected from both groups (60%). Multiple organisms were found from 4 specimens (enzymatic detergent arm = 1, chlorhexidine arm = 3).

CONCLUSION: The contamination rate of both types of cleaning solution is equivalent.

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Key words: Enzymatic detergent; Gastroscope; Bacterial decontamination

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INTRODUCTION

The endoscope is a complex, reusable device that requires reprocessing before being used in subsequent patients. Generally, a high-level of disinfection is required for reprocessing endoscopes^[1-2]. To date, all published incidents of pathogen transmission related to gastrointestinal (GI) endoscopy are associated with failure to follow established cleaning and disinfection/sterilization guidelines or with the use of defective equipments^[3-5].

Guidelines for reprocessing flexible gastrointestinal endoscopes have been recommended by several professional organizations^[6-10]. However, different professional organizations do not have similar recommended practices^[6-9]. Cleaning solutions are one of the different factors. In USA, multi-society guidelines for reprocessing flexible gastrointestinal endoscopes recommend to use enzymatic detergent as an initial endoscopic cleaning agent^[1]. However, different countries select different agents for this purpose. Chlorhexidine is one of the popular solutions that have been accepted for endoscope cleaning in Thailand. Unfortunately, there are some reports on bacterial transmission from this standard endoscope reprocessing practice^[7]. Bacterial biofilm is known to interfere with the cleaning efficacy of chlorhexidine. Biofilms consist of colonies of organisms forming structures to maximize growth potential. The ability of bacteria to form biofilms is an important factor in the pathogenesis of endoscopy-related infections, particularly as biofilms interfere with disinfection. Strategies aimed at decreasing biofilm formation and viability play an important role in endoscope disinfection because biofilms adhere to the internal channels of endoscopes^[4,7].

Recently, many professional organizations have accepted enzymatic detergent for endoscope cleaning^[1,7,9,11]. However, there is no randomized controlled study to demonstrate the efficacy of this agent for scope cleaning over chlorhexidine. Hence, the aim of this study was to evaluate the cleaning ability of these 2 agents combined with a standard disinfectant like glutaraldehyde to achieve high level disinfection for gastroscope cleaning.

MATERIALS AND METHODS

A prospective randomized controlled study was undertaken to evaluate the cleaning capacity of gastroscopereprocessing by 3E-ZYME (Medisafe UK Limited, Hartfordshire, UK) and hexene (Osoth Inter Laboratoreis, Chonburi, Thailand). All specimens were collected at the Gastroenterology Unit, King Chulalongkorn Memorial Hospital between July 2004 and October 2004. A total of 260 samples were collected from 5 different gastroscopes. These samples were divided into two groups by stratified randomization and block of 4. Group 1 ($n = 130$) received enzymatic detergent during endoscope cleaning, and group 2 ($n = 130$) received chlorhexidine detergent during endoscope cleaning.

The 3E-ZYME is a non-foaming, triple enzymatic detergent and designed for use in endoscope processing. It has a neutral pH formulation and is safe for instruments when used as directed. The directions indicate that 3E-ZYME should be diluted 3-7 milliliters (mL) to every liter (L) of warm (40°C-60°C) water and that the devices should be immersed for 1 min. In the other group, hexene was used as the conventional cleaning detergent. Hexene is an aqueous solution of 4% (weight/volume) chlorhexidine gluconate. In the present study, hexene was diluted from 25 mL to 5 L with filtered water, and the endoscopes were also immersed for 10 min.

Gastroscopereprocessing was performed in accordance with recognized standards for infection control and endoscope reprocessing. All personnel were well trained to comply with the protocol. The protocol for gastroscopereprocessing in the present study is shown in Table 1. Endoscope was randomly selected to be cleaned by one of the two cleaning agents. After gastroscopereprocessing was completely performed, a sample was collected by the flush method (injecting sterile water from the top of accessory channel of the endoscope and subsequently, the sample was collected from the distal tip of the endoscope). All samples were sent for aerobic bacterial cultures using a membrane filtering. Anaerobic bacterial, fungal and viral cultures were not performed due to insufficient information regarding the effect of bacterial biofilm over these organisms.

For quantitative culture, membrane filter method was performed in this study (limit of detection, 1 cfu/specimen). All inoculated plates were incubated aerobically at 37°C for 24-48 h before the number of colonies was counted. Culture results were variably reported as colony counts per milliliter. A colony count greater than 200 cfu/mL was considered significant.

Chulalongkorn University Institutional Board Review approved and supported all ethical issues related to this study. Descriptive statistics were expressed as n (%). Statistical analysis was performed by chi-square or Fisher's exact test. $P < 0.05$ was considered statistically significant. Data were analyzed with the Statistic of Package for Social Sciences (SPSS 11.5) program (Chicago, IL, USA).

RESULTS

All the five gastroscopes were equally distributed in the

Table 1 Steps for gastroscopereprocessing in the present study

Gastroscopereprocessing

Cleaning After completion of the cleaning procedure, the inserted tube was wiped with a wet cloth and soaked in detergent solution (chlorhexidine or 3E-ZYME). Detergent solution was suctioned through the biopsy channel until the solution was visibly clean.

While the scope was submerged, mechanical cleaning was performed by washing all debris from the exterior. All removable parts were separately cleaned. A soft cleaning brush was used to clean all accessible channels. Manual cleansing was done for 10 min.

The scope was removed from the detergent solution and then submerged in 5 L of filtered water. An all-channel irrigator was used to flush water through it.

Leak testing of the scope was performed.

Disinfection After manual cleaning, the gastroscopereprocessed high-level disinfection in a container using 2% glutaraldehyde with a 20-min soak time.

The scope was removed from 2% glutaraldehyde and then submerged in 5 L of filtered water. An all-channel irrigator was used to flush water through it.

Rinsing and Drying The suction/biopsy channel was rinsed with 70% alcohol 20 mL and dried for 5 min.

The suction/biopsy channel was sampled using the flush method.

Table 2 Characteristics of endoscopes in both groups

	Enzymatic detergent	Chlorhexidine
Specimen (n)	130	130
Endoscopes		
Olympus GIF-V	30	30
Olympus GIF-IT 140	30	30
Pentax 2970 K	35	35
Pentax 2930 K	22	22
Pentax 3830 TK	13	13

Table 3 Results of bacterial contamination after gastroscopereprocessing in both groups

	Enzymatic detergent ($n = 130$)	Chlorhexidine ($n = 130$)	P
Type of endoscope (Olympus:Pentax)	60:70	60:70	
Positive culture (> 200 cfu/mL)	6 (4.6%)	4 (3.1%)	0.747 ^a
Single organism	5 (3.8%)	1 (0.8%)	0.213 ^b
Mixed organism	1 (0.8%)	3 (2.3%)	0.622 ^b
<i>Pseudomonas aeruginosa</i>	4 (3.1%)	5 (3.8%)	1.000 ^b
Non <i>Pseudomonas</i> spp.	3 (2.3%)	3 (2.3%)	1.000 ^b

a: chi square test; b: Fisher's exact test.

2 groups (Table 2). The rates of bacterial contamination (> 200 cfu/mL) in both groups are shown in Table 3. The positive culture rate was 4.6% from the enzymatic detergent group and 3.1% from the chlorhexidine group. This was not statistically significant ($P = 0.747$).

Overall, the rate of bacterial contamination was 3.85% (10/260 samples). The incidences and types of organisms during study period are shown in Table 4. The most common organism was *Pseudomonas* (60%) in group 1 ($n = 4$, 3.1%) and group 2 ($n = 5$, 3.8%) (Table 3). Other organisms included *Klebsiella* species (13.33%), *Enterobacter* species (6.66%), *Acinetobacter baumannii*

(6.66%), *Staphylococcus coagulase negative* (6.66%) and *Staphylococcus aureus* (6.66%).

DISCUSSION

Ensuring safety in patients undergoing endoscopy, proper endoscope reprocessing is required. According to Spaulding classification of disinfection of medical and surgical instruments, flexible GI endoscope reprocessing is categorized as semicritical level since endoscopy has no involvement with tissue penetration^[12]. The reprocessing of endoscopes is susceptible to multiple errors, as it is a multi-step process relying on both human and material for reprocessing. The reprocessing involves meticulous manual cleaning and rinsing. This step is followed by high-level disinfection with liquid chemical germicide. Chlorhexidine is commonly used to decontaminate an endoscope prior to high level disinfection. However, recent reports from the Center of Disease Control and Prevention suggested that a significant number of infections are transmitted during endoscopic procedures after reprocessing these scopes under both manual and automated cleanings^[7]. Detailed analysis of these cases has identified either a breakdown in the cleaning process or a damage by equipment as the causative factor^[6,13]. It is possible that bacterial biofilm contributes to the failure of adequate endoscope reprocessing in certain instances. Vickery *et al*^[14] showed that bacterial biofilm is an important factor in endoscope contamination, and that routine cleaning procedures do not remove biofilm reliably from endoscope channels. Generally, biofilm consisting of bacteria enclosed in a matrix of exopolysaccharide (EPS) can form on many medical devices such as catheters and endoscopes. Chemical cleaning methods by agents like chlorhexidine are often ineffective because biofilm has a strong resistance to these biocides. Biofilm removal by physical methods such as ultrasound and mechanical cleaning is reasonably effective but it is difficult to supervise in practice.

To solve this problem, agents that can be used to remove the bacterial biofilm during the process of endoscope cleaning are desirable. The efficacy of enzymatic cleaning agents to reduce the bacterial load and biofilm in laboratory setting has been studied recently^[15]. In addition, the ASGE and the SHEA have recently endorsed enzymatic detergents in reprocessing endoscopes and reusable accessories^[1].

Enzymatic detergents generally containing various combinations of protease, lipase and amylase, require a minimum contact time to enable them to adequately remove the bacterial biofilm^[16]. To date, there has been no report on the bacterial decontamination rate of these enzymatic detergents for endoscope reprocessing.

The bacterial concentration cultured from an endoscope after decontamination is an important factor in determining the risk of transmission from an endoscope to a patient. At present there is no standard bacterial concentration above which the endoscope is considered contaminated. We used the AAMI^[17,18] guidelines established for hemodialysis water, < 200 cfu/mL.

In our series, the overall rate of bacterial contamination above the cut off level from enzymatic detergent and

Table 4 Incidence and types of organisms during study period

Type of organism	Enzymatic detergent (samples, n)	Chlorhexidine (samples, n)	Total, n (%)
<i>Pseudomonas aeruginosa</i>	4	5	9 (60)
<i>Klebsiella</i> species	1	1	2 (13.3)
<i>Enterobacter</i> species	1	0	1 (6.7)
<i>Acinetobacter baumannii</i>	0	1	1 (6.7)
<i>Staphylococcus coagulase negative</i>	1	0	1 (6.7)
<i>Staphylococcus aureus</i>	0	1	1 (6.7)
Total	7	8	15 (100)

chlorhexidine was very low (3.85%). This is significantly different from previous studies that mainly used non-enzymatic cleaning agents which demonstrated a contamination rate as high as 24%^[1,5]. The majority of bacteria identified in this study were Gram-negative bacilli. *Pseudomonas aeruginosa* was the most common species. This is similar to other published series^[19,20]. Though we did not observe any adverse clinical outcomes in the patients exposed to the contaminated endoscopes, the primary goals of this study were not to address this question.

A group from Walter Reed Army Medical Center reported that their surveillance of bacterial culture result from GI endoscopes is as high as 14.5% and that more than half of positive cultures are obtained from therapeutic scopes that were used during emergency procedure, which might be attributed to faulty mechanical cleaning by non-nursing personnel after emergent procedures^[5]. Furthermore, adherence to the standard guideline for endoscope reprocessing can result in a low rate of disease transmission^[1].

In conclusion, 4% chlorhexidine is not worse than enzymatic detergent for endoscope decontamination. Both of them have a very low rate of significant positive bacterial cultures. Further investigations on the effectiveness of the enzymatic agent on decontamination of other organisms apart from aerobic bacteria are required.

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Biochemical response to lamivudine treatment in HBeAg negative chronic hepatitis B patients in Iran

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Abstract

AIM: To study the effect of a one-year lamivudine regimen in patients with chronic hepatitis B.

METHODS: Medical records of HBeAg negative hepatitis B patients who attended a hepatitis clinic in Tehran between March 2002-March 2004 were evaluated. The patients received 100 mg lamivudine tablets once daily for at least 12 mo. Liver enzymes and complete blood count were checked at baseline and the end of treatment (12th mo) and 6 mo after discontinuation of treatment.

RESULTS: Of all patients, 24 were excluded. Of 71 patients left, 58 (81.7%) were men. Mean age of the patients was 38 ± 14 years. Mean level of ALT in serum was 1437 ± 205 nkat/L at baseline with a significant reduction at the end of treatment to a mean level of 723 ± 92 nkat/L ($P = 0.002$). In 38 patients (53.5%), the ALT level was normal after one-year treatment. Five patients (7.3%) relapsed (biochemically) within 6 mo after discontinuing lamivudine therapy (the patients with good end of treatment response). Mean level of AST in serum was 1060 ± 105 nkat/L at baseline which decreased significantly to 652 ± 75 nkat/L at the end of treatment ($P = 0.002$).

CONCLUSION: Over half (53.5%) of chronic hepatitis B patients with HBeAg negative have normal liver enzyme level at 12-mo lamivudine therapy.

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Key words: Chronic hepatitis B; Lamivudine; HBeAg

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INTRODUCTION

Chronic hepatitis B is a pandemic disease which has been under constant focus because it has long-term serious complications such as hepatic cirrhosis and hepatocellular carcinoma, while it is a preventable condition. About 350 million people have the condition all around the world with 200 000 patients living in Iran^[1]. Length of drug therapy, frequency of recurrences after primary remission, cost of drug regimen and resistance to treatment have led investigators to look for the best and most cost-effective therapeutic regimens for chronic hepatitis B in numerous studies throughout the world. A study in Canada estimated the annual cost of medical care for a patient with chronic hepatitis as high as \$2191 while a cirrhotic patient's medical care costs summed up to an estimated \$11 228 each year^[2].

The objectives of treatment in chronic hepatitis B patients are suppression of viral replication as well as reduction in hepatic inflammation. Lamivudine is a nucleoside analogue which prevents the viral DNA synthesis. Markers such as HBeAg, liver enzymes especially ALT and HBV DNA are used for monitoring patients' response to treatment. Many studies assessed the effectiveness of a number of different therapeutic regimens in different community contexts^[3,4]. In the present study, we investigated the effect of a one-year lamivudine regimen in HBeAg negative patients with chronic hepatitis B who attended a hepatitis clinic in Tehran.

MATERIALS AND METHODS

Patients

Ninety-five medical records of HBeAg negative hepatitis B patients who were registered in a hepatitis clinic in Tehran during March 2002 to March 2004 were evaluated; these patients had positive HBsAg, positive HBeAb, negative HBeAg and sustained or intermittent increased ALT for more than 6 mo. Serum HBV DNA loads were measured by quantitative real-time PCR with primers that amplify

Table 1 Frequency of response and serum liver enzymes during 12-mo treatment of HBeAg negative patients with lamivudine ($n = 71$)

	Baseline	3 mo		6 mo		12 mo	
		Mean	Frequency of response	Mean	Frequency of response	Mean	Frequency of response
ALT (nkat/L)	1437 ± 205	1060 ± 122	65 (91.5%)	813 ± 160	61 (85.9%)	723 ± 92	38 (53.5%)
AST (nkat/L)	1060 ± 105	960 ± 77	68 (95.7%)	770 ± 88	63 (88.7%)	652 ± 75	36 (50.1%)

$P = 0.002$ for ALT between baseline and 12 mo treatment levels; $P = 0.002$ for AST between baseline and 12 mo treatment levels.

a highly conserved region of the surface gene^[5] and with a detection system that employed molecular beacon technology^[6]. The assay was calibrated using international HBV standards and a plasmid control containing a full-length cloned copy of HBV ayw. The assay has a 7 log₁₀ linear response, with a lower limit of detection of 2×10^5 copies/L and an intraassay coefficient of variability of 15%. All patients with a serum ALT level of more than 2 times of upper normal limit, serum HBV DNA more than 10^5 copies/mL with histopathologic features in liver biopsy consistent with chronic hepatitis (\geq F2) in the Knodell score, [also known as the histologic activity index (HAI)], were included. Those who had evidence of other liver diseases such as hepatitis C, autoimmune liver diseases, non alcoholic fatty liver disease (NAFLD) and non alcoholic steatohepatitis (NASH) in sonography and liver biopsy, and biliary tract diseases, thyroid diseases were excluded. Also, all cases of non-compliance with therapy were excluded.

Methods

Lamivudine was prescribed at a dosage of 100 mg daily. All patients had used the drug for at least 12 mo. Liver enzymes (AST, ALT), complete blood cell counts were checked and recorded at baseline, mo 3 and 6 of treatment, the end of one year treatment and 6 mo after discontinuation of treatment. Forty-two patients had detected serum HBV DNA loads at the end of treatment for their own interest in evaluation of some virological responses.

Statistical analysis

The data were analyzed by SPSS and paired t test was used to compare the variables before and after treatment. $P < 0.05$ was taken as significant.

RESULTS

Of all medical records, 24 were withdrawn from the study: 16 patients did not take the prescribed drug correctly, and 8 patients had a follow-up period of less than 6 mo. Of 71 patients left, 58 were men. Mean age of the patients was 38 ± 14 years. The youngest patient was 12 years old while the oldest was 71. Mean weight of patients was 68 ± 14 kg. The lowest weight was 38 kg while the highest was 115 kg. Biochemical markers are as follows: Mean level of hemoglobin was 144 ± 17 g/L with lowest value of 109 g/L and highest value of 175 g/L. Mean white blood cells count was $(6.39 \pm 2.2) \times 10^9$ /L with lowest count of 3.1×10^9 /L and highest count of 11.6×10^9 /L. Mean prothrombin time was 14.3 ± 4.5 s at baseline with

Table 2 Liver serum markers after 3 and 6 mo of termination of one-year regimen in patients who responded to treatment ($n = 38$)

	12 mo Mean	After 3 mo		After 6 mo	
		Mean	Frequency of normal level	Mean	Frequency of normal level
ALT (nkat/L)	538 ± 90	688 ± 157	28 (73.7%)	902 ± 238	25 (65.8%)
AST (nkat/L)	447 ± 122	677 ± 143	32 (84.2%)	803 ± 210	28 (73.7%)

$P < 0.04$ between 12 mo of and after 6 mo cessation of treatment for ALT, $P < 0.03$ between 12 mo of and after 6 mo cessation of treatment for AST.

shortest time of 11 s and longest time of 46 s. Mean platelet count was $(199.9 \pm 9.03) \times 10^9$ /L. Of all patients, 20 (28.2%) were diagnosed by clinical manifestation while a major proportion of patients (32%-45.0%) were diagnosed incidentally by routine examination on the blood they donated. Only 7 patients were diagnosed through screening and checkups.

In terms of probable routes of transmission, 43.7% had a history of dental procedure, 15.5% had a tattoo and 12.7% underwent a surgical procedure. Of all patients, 44 (62%) had no family history of hepatic disease. Those who had a positive family history had a sibling with the condition, while 4 mothers and 8 fathers had the condition.

A biopsy was taken from 37 patients before the treatment. The biopsy samples were reported based on Knodell score. Of these samples, 73% had a score of less than 6. The liver enzyme levels before, during and after treatment with lamivudine were compared. As shown in Table 1, mean level of ALT in serum was 1437 ± 205 nkat/L at baseline with a significant reduction after treatment to a mean level of 723 ± 92 nkat/L ($P = 0.002$). In 53.5% of patients the ALT level was normal after one-year treatment. Mean level of AST in serum was 1060 ± 105 nkat/L at baseline which decreased significantly to 652 ± 75 nkat/L after treatment ($P = 0.002$). Of 38 (53.5%) patients who responded to treatment, 28 (73.7%) and 25 (65.8%) patients had normal ALT levels after 3 and 6 mo of termination of one-year regimen, respectively. There was no significant elevation of ALT and AST after 3 mo of cessation of treatment compared to 12 mo of treatment, while there was a significant difference between ALT and AST level at 6 mo after termination of and 12 mo of treatment ($P < 0.04$ and $P < 0.03$, respectively, Table 2). At the end of treatment, HBV DNAs were measured in 42 cases and was negative in 24 cases.

Mild adverse events were seen in 12 patients including fatigue in five patients, headache in three, myalgia in two, decreased appetite in two, but there were no reports of therapy discontinuation.

DISCUSSION

The major proportion of the patients were men and the age distribution of sample can be regarded as an adult population. The probable routes of transmission in order of frequency were a history of dental procedure, having a tattoo and a history of surgical procedure, which is consistent with other studies^[7,8]. However, to confirm these results, studies to evaluate the precise role of these risk factors in the transmission of hepatitis B are needed. It is clear that the risk of transmission of hepatitis B *via* these procedures has not been evaluated completely in our study. The most frequent event leading to diagnosis was blood donation, which is consistent with other studies^[9]. The most significant factor under focus in our study was the changes in serum levels of liver enzyme especially ALT after treatment by lamivudine. As presented earlier the reduction in serum level of ALK, AST, and ALT were significant at 12 mo of therapy.

In a 3-year retrospective study in the United States, the therapeutic response markers of 119 patients were analyzed. The results showed that 61% of patients had a normal ALT level after treatment. The ALT serum level at baseline was not a significant predictor for response to therapy by lamivudine^[10]. Similar results were reported by a Chinese study on 129 patients with chronic hepatitis. In the current study 60.3% of patients completing a one-year lamivudine treatment had a normal ALT level while the ALT level was normal in only 24.5% of controls at 12 mo. The difference was significant^[11]. A recent study in Pakistan showed that a course of one-year lamivudine treatment was associated with significant reduction in ALT serum level, the treatment was more effective in patients with HBeAg at baseline^[12]. In another study in Austria, 72% of the intervention group had normal ALT level while 29% of controls had normal ALT level^[13]. Most studies of one-year lamivudine treatment for patients with chronic hepatitis B showed a reduction of liver enzyme to normal level at 12 mo^[14-18]. In our study, 53.5% of patients had normal liver enzyme level at 12 mo of lamivudine therapy, which is slightly lower than that of other studies.

In patients whose enzyme level was not normal after lamivudine treatment (50% of patients), it is likely that a resistant mutant is present which warrants further study for confirmation.

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RAPID COMMUNICATION

Comparison of invasive methods and two different stool antigen tests for diagnosis of *H pylori* infection in patients with gastric bleeding

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Abstract

AIM: To compare two different *H pylori* stool antigen tests as noninvasive diagnostic methods.

METHODS: The study population consisted of 22 upper gastrointestinal system bleeding patients. Urea breath test (UBT), rapid urease test (RUT) and histopathological examination were applied to all patients. Stool specimens from these patients were examined by rapid STRİP!HpSA and one step simple *H pylori* antigen cassette test for the detection of *H pylori* antigens.

RESULTS: For these 22 patients, 15 (68.2%) were diagnosed as positive and seven (31.8%) were diagnosed negative for *H pylori* infection by the gold standard methods. Whereas 10 (45.5%) were positive and 12 (54.5%) were diagnosed negative by the rapid STRİP!HpSA test. The sensitivity, specificity, positive and negative predictive values were 60%, 86%, 90% and 50%, respectively. When compared to the gold standard methods, these differences were not significant. However, six patients (27.3%) were positive, and 16 (72.7%) were negative by the simple *H pylori* stool antigen cassette test. The sensitivity, specificity, positive and negative predictive values were 33%, 86%, 83% and 38%, respectively. Compared to the gold standard methods, the simple *H pylori* stool antigen cassette test results were significantly different ($P = 0.012$).

CONCLUSION: Rapid STRİP!HpSA test could be used as a routine diagnostic tool in the microbiology laboratory for assessing clinical significance and eradication control of *H pylori* in upper gastrointestinal system bleeding patients.

INTRODUCTION

H pylori is a microaerophilic gram-negative spiral shaped bacterium^[1-3]. It is recognized as the major cause of gastritis, gastric and duodenal ulcers, gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma, however little is known about its role in functional dyspepsia^[4,5]. *H pylori* colonizes the gastric mucosa and attaches to the gastric epithelial cells^[6]. The prevalence of *H pylori* infection is 70%-90% in developing countries and 25%-50% in developed countries^[2,7]. Person-to-person spread is the most probable mode of transmission. Faecal-oral and oral-oral transmissions have been reported^[8]. Chronic *H pylori* infections of the stomach are increasingly recognized as a major risk factor for the development of gastroduodenal disease. *H pylori* can be detected by noninvasive and invasive methods, the latter requiring endoscopy. Noninvasive testing for *H pylori* can be done by measuring exhaled ¹³C urea breath test (UBT), by serology, by stool antigen tests, by a simple stool PCR and by analyzing body materials such as saliva and urine^[2,9]. Although histopathology and culture of the organism, which are not easily and routinely performed, is considered the gold standard for the diagnosis of *H pylori* infection, we need rapid, accurate and reliable noninvasive methods^[2].

In the case of upper gastrointestinal bleeding which is a major cause of morbidity, mortality and medical care costs, peptic ulcer is the most frequent source of bleeding in these patients. Treatment of *H pylori* infection is more effective than antisecretory non-eradicating therapy in preventing recurrent upper gastrointestinal bleeding from peptic ulcer. Consequently, all patients with peptic ulcer

bleeding should be tested for *H pylori*, and eradication therapy should be prescribed to infected patients^[10].

In the presence of upper gastrointestinal bleeding, the diagnosis of *H pylori* infection may be compromised. The UBT is responsible for a high number of false negative results when it is used to diagnose *H pylori* in patients with upper gastrointestinal bleeding. Coagulation disorders or anticoagulation may prevent a biopsy being taken. The UBT may not be feasible in patients on artificial respiration, or in the presence of impaired consciousness or acute abdominal disease. Therefore, it is suggested that noninvasive methods, such as serology or UBT, be used to identify *H pylori* infection in these patients. In some cases, the indication for *H pylori* eradication therapy is based only on a serological test. Serology alone, however, is a rather inaccurate diagnosis method^[10-12]. An ideal noninvasive test for *H pylori* infection should be safe and acceptable to patients, inexpensive and easy to perform, and with a high degree of sensitivity and specificity^[13].

The diagnostic role of HpSA test and simple *H pylori* antigen cassette test in patients with upper gastrointestinal bleeding remains unclear. Only a few reports have discussed the results of the HpSA test and simple *H pylori* antigen cassette test in these patients^[10-12,14]. The aim of this study was to evaluate diagnostic accuracy of stool antigen tests of rapid STRIP!HpSA and simple *H pylori* antigen cassette test other than HpSA in patients with upper gastrointestinal bleeding. We also compared two different *H pylori* stool antigen tests as noninvasive diagnostic methods with UBT, RUT and histopathology as gold standard methods.

MATERIALS AND METHODS

Patients

The study population consisted of 22 upper gastrointestinal system bleeding patients (13 males, 9 females; mean age, 58 ± 18 years; age range, 20 to 86 years) between September 2004 and January 2005. UBT (Infai, Germany), rapid urease test (RUT) and histopathological examination were applied to all patients.

The diagnosis of *H pylori* was defined as positive for UBT alone or for histopathology and (or) RUT results defined as gold standards. A patient was classified as *H pylori* negative when UBT and (or) histopathological examination and urease test were both negative.

Endoscopy and biopsy sampling

Two antrum and two corpus biopsy specimens were taken from each patient undergoing upper endoscopy from the same location in the stomach: one from the antrum and the corpus. One of these was used for the rapid urease test and the other two were immediately fixed and transported in 10% phosphate-buffered formalin solution for histopathologic examination.

Histopathologic examination of biopsy specimens

Paraffin-embedded gastric biopsy specimens were routinely processed. Haematoxylin-eosin, Alcian blue and Giemsa stains were used for morphologic examination

of *Helicobacter*-like organisms (HLO) and updated Sydney system was used^[15]. Histopathology was performed by a specialized pathologist.

Stool specimens

Stool specimens from these patients were collected and were kept at -20°C until used. They were examined by rapid STRIP!HpSA (Meridian Bioscience Europe) and one step simple *H pylori* antigen cassette test (Linear Chemicals, S.L. Spain) for the detection of *H pylori* antigens. Both tests were performed in accordance with the manufacturer's specifications.

Stool antigen tests

Rapid STRIP!HpSA (Meridian Bioscience Europe) is a rapid immunoassay using a monoclonal anti-*H pylori* antibody on a strip for the detection of *H pylori* infections in stool specimens. The strip is introduced in a tube containing diluted patient samples and the appearance of a pink-red line in the reading area indicates a positive result after 5 min of incubation at room temperature. A positive test result is evaluated according to the blue band (control line), a distinguishable pink-red band (test line) also appears across the white central zone of the reaction strip. Any pink-red line, even very weak, must be considered as a positive result. By contrast, the sample is considered negative when only one blue coloured band (control line) appears across the white central area of the reaction strip.

Simple *H pylori* antigen cassette test (Linear Chemicals, S.L. Spain) is a rapid immunochromatographic test using a single monoclonal antibody. A diluted fecal sample is placed in an immunochromatographic support and the result is read after 5 min. A positive test will display a red line in the reading area next to a blue control line. Even a minimal trace of a red line was considered positive. By contrast, the sample is considered negative when only the control blue line develops. If no line appears in the reading area the test is considered null^[14,16].

RESULTS

The diagnosis of *H pylori* was defined as positive for UBT alone or for histopathology and (or) RUT results defined by gold standard methods. A patient was classified as *H pylori* negative when histopathological examination and urease test were both negative.

For those 22 patients, 15 (68.2%) were diagnosed as positive and seven (31.8%) negative for *H pylori* infection by the gold standard methods. Meanwhile, 10 (45.5%) were positive and 12 (54.5%) were negative by the rapid STRIP!HpSA test. The sensitivity, specificity, positive and negative predictive values were 60%, 86%, 90% and 50%, respectively. When compared to the gold standard methods, these differences were not statistically significant. However, six patients (27.3%) were positive, and 16 (72.7%) were negative by the simple *H pylori* stool antigen cassette test. The sensitivity, specificity, positive and negative predictive values were 33%, 86%, 83% and 38%, respectively. Compared to the results by the gold standard methods, the simple *H pylori* stool antigen cassette test

Table 1 Results of invasive and noninvasive methods for diagnosis of *H pylori* infection in patients with gastric bleeding

	Positive n (%)	Negative n (%)	False positive n (%)	False negative n (%)
Urea breath test	12 (54.54)	10 (45.45)	2 (9.09)	2 (9.09)
Rapid urease test and/or histopathology	15 (68.18)	7 (31.82)	-	-
Rapid STRIP!HpSA stool antigen test	10 (45.45)	12 (54.54)	1 (4.54)	6 (27.27)
Simple <i>H pylori</i> stool antigen cassette test	6 (27.27)	16 (72.73)	1 (4.54)	10 (45.45)

results were statistically different ($P = 0.012$) (Table 1).

DISCUSSION

Many studies have described the use of ELISA-based HpSA stool antigen kits with either polyclonal or monoclonal antibodies, for diagnosis of *H pylori* infections. In this study we evaluated the diagnostic accuracy of two non-ELISA-based kits, rapid STRIP!HpSA and simple *H pylori* antigen cassette, in patients with upper gastrointestinal bleeding.

Meridian Bioscience, Inc. introduced the concept of detecting *H pylori* antigens in stool specimens, with a microtiter based immunoassay, in 1997. Primer Platinum HpSA, after extensive evaluation, was accepted as an accurate tool for non-invasive *H pylori* infection diagnosis^[17-19]. Recent official European Guidelines recommend the use of either stool antigen or urea breath test for diagnosis and confirmation of eradication four weeks after the end of the treatment^[20].

To our knowledge, three recent studies have evaluated the HpSA test in the presence of gastrointestinal bleeding: Gisbert *et al* ($n = 34$ hospitalized patients; sensitivity of polyclonal ELISA, monoclonal ELISA and monoclonal immunochromatographic test was 74%, 94%, 60%, respectively), Lin *et al* ($n = 93$ patients with bleeding peptic ulcers and 59 patients with nonbleeding peptic ulcers; sensitivity 82% and specificity 68%) and Peitz *et al* ($n = 114$ patients; the sensitivity 84% and specificity 90%)^[10-12].

In another study, Erzin *et al* compared two different stool antigen tests for the primary diagnosis of *H pylori* infection in Turkish patients with dyspepsia. A total of 151 patients who were referred to the endoscopy unit were included. They used FemtoLab *H pylori* enzyme immunoassay and Premier Platinum. The sensitivity and specificity of the monoclonal FemtoLab *H pylori* were 93% and 90% respectively, and of the polyclonal Premier Platinum HpSA were 84% and 67%, respectively. They concluded that Femtolab *H pylori* was an excellent tool for primary diagnosis of *H pylori* in Turkish patients with dyspepsia^[21].

Still in another study, Kato *et al* compared rapid lateral flow stool antigen immunoassay (LFI) and stool antigen enzyme immunoassay (EIA) for the diagnosis of *H pylori* infection in children. One hundred and eighty-two children and adolescents were studied. The sensitivity, specificity and accuracy of the LFI method were 90.6%, 95.8% and

94% respectively and for the EIA method, sensitivity, specificity and accuracy were 96.8%, 99.2% and 98.3%, respectively^[22].

Islam *et al* assessed the performance of the HpSA for the diagnosis of *H pylori* infection and confirming post-therapy eradication compared to generally well accepted clinical reference standard. HpSA was used for the 127 patients. The sensitivity and specificity of HpSA were 67% and 100%, respectively. As a result, HpSA was found to be a reasonably useful diagnostic test for *H pylori*. The HpSA may prove to be useful for the primary care physicians as part of the test-and-treat strategy for dyspepsia, but this may need further study^[23].

Ito *et al* investigated the clinical usefulness of HpSA test for the evaluation of the success of eradication therapy by comparing it with the UBT. A total of 105 patients with *H pylori* infection were enrolled in that study. The diagnostic accuracy of the UBT and the HpSA test was 94.3% and 97.1%, respectively. As a result, HpSA is a very useful and non-invasive diagnostic tool for the evaluation of eradication therapy of *H pylori*. A combination of the HpSA test and the UBT is very practical in the clinical evaluation of eradication therapy of *H pylori*^[24].

Manes *et al* compared the accuracy of HpSA, FemtoLab and UBT in the assessment of eradication of *H pylori* infection 4-8 wk after the completion of antibiotic treatment. Three hundred and forty-six patients were studied. The sensitivity and specificity of HpSA were 73.4% and 97.8%, respectively. The sensitivity and specificity of FemtoLab were 88.3% and 97.8%, respectively. They concluded that both the new stool antigen tests, although less accurate, may represent valuable alternatives to UBT since they were cheap and easy to perform and did not need the use of expensive isotope ratio mass spectrometers. Thus, due to its high level of sensitivity, the new monoclonal stool test could be preferred for the post-eradication setting of *H pylori* infection^[25].

Five recent studies done by Shaikh *et al* ($n = 86$ children; sensitivity 76% and specificity 61%), Raguza *et al* (133 children; sensitivity 94.6% and specificity 96.6%), de Carvalho Costa Cardinali *et al* (161 children; sensitivity 96.9% and specificity 100%), van Doorn *et al* (106 children; sensitivity 100% and specificity 92%) and Elitsur *et al* (121 children; sensitivity 67% and specificity 99%) evaluated the HpSA test in asymptomatic children with *H pylori* infection compared to other methods. It was demonstrated that the commercial polyclonal HpSA test can not replace histopathologic findings as the best standard for the diagnosis of *H pylori* infection in children^[26-30].

Syam *et al* evaluated the HpSA for the detection of *H pylori* infection in 63 dyspeptic patients. The sensitivity and specificity of HpSA were 66.7% and 78.9%, respectively. They concluded that HpSA stool test may be useful for the primary diagnosis of *H pylori* infection in peptic ulcer^[31].

Arikan *et al* conducted a prospective study to examine the reliability of the HpSA test. The HpSA test had a 91% sensitivity and 83% specificity. HpSA test proved to be as reliable as pathological examination for confirming the existence of *H pylori* in humans. Thus, the HpSA test was a useful method for detecting *H pylori* in patients for whom

endoscopy was not indicated^[32].

Lopez *et al* compared the efficacy of several non-invasive methods and assessed comparative reliability of the stool tests. Eighty-six patients were applied FemtoLab, HpSA and Simple *H pylori* tests. The sensitivity and specificity of HpSA were 58% and 96%, respectively and the sensitivity and specificity of simple *H pylori* were 61% and 78%, respectively. According to the results, they suggested that UBT was the most reliable diagnostic examination for determining *H pylori* status in patients with chronic renal failure on dialysis because stool tests showed heterogeneous results^[14].

Inelmen *et al* evaluated the accuracy of HpSA in the diagnosis of *H pylori* infection in 85 elderly patients affected by medication. Among 56 patients who were not taking PPIs, the sensitivity and specificity of the HpSA test were 76% and 93%, respectively. Among 29 patients who had received pharmacological therapy with PPIs, the sensitivity and specificity of HpSA test were, respectively, 82% and 83%. They concluded that HpSA was a useful test in elderly people. The test was easy, simple to perform and non-invasive^[13].

Chisholm *et al* conducted a comparative evaluation of the performances of Premier Platinum HpSA ELISA, Amplified IDEIA HpStAR ELISA and ImmunoCard STAT!HpSA kits. ImmunoCard STAT!HpSA was demonstrated easier to perform than ELISA and was more sensitive than the HpSA kit. Compared with the IDEIA HpStAR kit, the ImmunoCard test was less sensitive (87.8% versus 95.9%, respectively) and specific (89.4% versus 100.0%, respectively). The Amplified IDEIA HpStAR kit was the most sensitive and specific of the three tests and it was available for pre-treatment, noninvasive detection of *H pylori* in stool samples in an English adult dyspeptic population^[33].

Previously we studied forty adult Turkish dyspeptic patients. Two antrum and corpus biopsies were taken from each patient and RUT and histopathology were applied to all patients as gold standard methods for the diagnosis of *H pylori* infection. Stool specimens were examined by polyclonal Premier Platinum HpSA and simple *H pylori* cassette test before and after the eradication therapy. When we compared with gold standard methods, the diagnostic accuracy of Premier Platinum HpSA and simple *H pylori* cassette tests were 75% and 87.5%, respectively.

In that study the HpSA test was polyclonal test but we wanted to compare it with the available monoclonal Simple *H pylori* cassette test^[34]. The new Rapid STRIP!HpSA test was also used in this group of patients (unpublished data). As no results have been published to date on the rapid STRIP!HpSA test and the simple *H pylori* stool antigen cassette test, we undertook the present study. The simple *H pylori* stool antigen cassette test, although easy to use, gave a sensitivity that was too low (33%) for the reliable diagnosis of *H pylori* infection. However, the rapid STRIP!HpSA test, which was also convenient to use, was more sensitive (60%). We conclude that because it is a monoclonal test, rapid STRIP!HpSA, can be used as a routine diagnostic tool in the microbiology laboratory for assessing clinical significance and eradication control of *H pylori* in upper gastrointestinal bleeding patients.

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Incidence and management of colonoscopic perforations: 8 years' experience

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Abstract

AIM: To review the experience of a major medical teaching center with diagnostic and therapeutic colonoscopies and to assess the incidence and management of related colonic perforations.

METHODS: All colonoscopies performed between January 1994 and December 2001 were studied. Data on patients, colonoscopic reports and procedure-related complications were collected from the departmental computerized database. The medical records of the patients with post procedural colonic perforation were reviewed.

RESULTS: A total of 12 067 colonoscopies were performed during the 8 years of the study. Seven colonoscopic perforations (4 females, 3 males) were diagnosed (0.058%). Five occurred during diagnostic and two during therapeutic colonoscopy. Six were suspected during or immediately after colonoscopy. All except one had signs of diffuse tenderness and underwent immediate operation with primary repair done in 4 patients. No deaths were reported.

CONCLUSION: Perforation rate during colonoscopy is low. Nevertheless, it is a serious complication and its early recognition and treatment are essential to optimize outcome. In patients with diffuse peritonitis early operative intervention makes primary repair a safe option.

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Key words: Colonoscopy; Complications; Perforation; Polypectomy; Management

INTRODUCTION

Since first introduction in 1969^[1] flexible colonoscopy has been accepted as the best method for the diagnosis, treatment and follow-up of colorectal pathologies. Nevertheless, being an invasive procedure it harbors major risks of bleeding, perforation and even death^[2-4]. The incidence of perforation is 0.2% to 0.4% for diagnostic colonoscopy and 0.3% to 1.0% with polypectomy^[2,5,6]. Recent large series have reported lower perforation rates of 0.002% to 0.19%^[7-10]. The aim of the present study was to review the experience of a major university affiliated medical center with colonoscopy and to assess the incidence of perforations and their management.

MATERIALS AND METHODS

A total of 12067 colonoscopies were performed between January 1994 and December 2001. Data on patients undergoing colonoscopy was entered into a computerized database and included demographic patient information and detailed colonoscopic reports. A retrospective review of the medical records of all patients diagnosed with colonic perforation after colonoscopy was performed. The following parameters were analyzed: patient age and sex, background disease, laboratory work-up, indication to endoscopy, interval from the procedure to the diagnosis of perforation, clinical presentation, location, management, and outcome of the perforation.

RESULTS

Of 12 067 colonoscopies performed, seven were associated with colonic perforation. There were 4 women and 3 men with a mean age of 70 years (range 31-80) (Table 1). The procedure was done on an outpatient basis in all cases. Five perforations occurred during diagnostic colonoscopy, one during polypectomy and one during electrocoagulation of an arteriovenous malformation. Six perforations were

identified during the procedure or immediately thereafter, and one patient who had a cecal polyp coagulated with hot biopsy forceps, was diagnosed about 24 h after the procedure. In 3 patients perforation was suspected when a hole in the intestinal wall was noted. All the patients had severe abdominal pain and distention. Plain abdominal roentgenograms performed in all 7 patients showed free intraperitoneal air in 4, retroperitoneum in 2 and no abnormalities in one patient.

Six patients had, on examination, diffuse abdominal tenderness and underwent immediate operation. All five perforations that occurred during diagnostic colonoscopy were found to be in the sigmoid colon, and repair was achieved mostly by debridement and primary suture of the perforated site. The postoperative period was uneventful. One patient was treated nonoperatively with intravenous fluids, antibiotics and intestinal rest and was placed under close clinical observation. He was afebrile and had localized abdominal tenderness with no peritoneal signs or leukocytosis. The patient who was diagnosed and operated on about 24 h post colonoscopy had a wound infection. Median hospital stay of the operated group was 8 d (range 4-15). The patient who was treated nonoperatively was hospitalized for 13 d. There were no deaths.

DISCUSSION

Colonic perforation occurs rarely during colonoscopy but it is still considered a major complication^[2-4]. During the 8-year period reviewed, there was a combined diagnostic and therapeutic colonoscopic perforation rate of 0.058%. This rate is lower than that in most of the published series^[7-10]. The management of colonoscopic perforations may be conservative or surgical, and should be selective. The choice of treatment depends on the mechanism and size of the perforation, adequacy of bowel preparation, timing of diagnosis, the patient's clinical condition and the primary colonic pathology^[9-16]. Table 1 summarizes some reports evaluating the incidence and management of colonoscopic perforations. The low morbidity rate in our series is probably attributed to the combination of supportive treatment and early surgical intervention, which resulted in no intraperitoneal contamination in 4 out of 5 patients, and therefore primary repair could be completed safely. Early surgical exploration in all patients with peritoneal irritation or free air on abdominal X-ray is recommended by other authors as well^[10,17,18]. Farley *et al*^[10] reported on 43 perforations among 57 028 colonoscopies (0.075%). Forty-two were treated by emergency laparotomy. Most patients underwent primary repair or limited resection with anastomosis. The authors concluded that in order to minimize morbidity and mortality prompt operative intervention is the best strategy in most patients. Dafnis *et al*^[18] reported on 8 perforations in 6066 colonoscopies (0.13%). All patients underwent surgery. Most perforations were repaired by primary closure, and the postoperative course was uneventful in all patients.

Perforations occurring during diagnostic colonoscopy are due to direct mechanical penetration with the instrument tip, sharp flexion of the colonoscope, high pressure applied when a loop is formed or barotrauma

Table 1 Reported colonoscopic perforation rates and management

Author	Time interval (yr)	No. of colono-scopies	Perforation	Mortality n (%)	
				Operative	Non op.
Anderson <i>et al</i> ^[7]	10	10 486	20 (0.19)	19 (1)	1 (1)
Araghizadeh <i>et al</i> ^[9]	30	34 620	31 (0.09)	20 (0)	11 (1) ¹
Farley <i>et al</i> ^[10]	16	57 028	43 + 2 ² (0.075)	42 (0)	3 (0)
Christie <i>et al</i> ^[12]	10	4784	7 (0.15)	2 (0)	5 (0)
Hall <i>et al</i> ^[14]	6	17 500	15 (0.09)	14 (0)	1 (0)
	(4-15)				
Jentschura <i>et al</i> ^[16]	9	29 695	31 (0.1)	24 (2)	7 (0)
Lo <i>et al</i> ^[17]	6	26 708	12 (0.04)	6 (1)	6 (0)
Dafnis <i>et al</i> ^[18]	17	6066	8 (0.1)	8 (0)	0
Carpio <i>et al</i> ^[25]	10	5424	14 (0.26)	8 (2)	6 (1)
Present study	8	12 067	7 (0.058)	6 (0)	1 (0)

¹Three of 11 patients failed medical treatment and required surgery; ²Two patients were treated after colonoscopy performed elsewhere; ³Includes only colonoscopic polypectomies.

as a result of aggressive gas insufflation^[19,20]. The most common underlying cause in the present study was direct mechanical injury of the colonic wall by the colonoscope. It occurred in patients with diverticular disease or a strictured, severely diseased colonic segment. These risk factors are in accordance with those noted in the literature^[21,22]. Perforations during therapeutic colonoscopy occur as a result of similar mechanisms, as well as from thermal or electrical injury, as in two cases in the present study^[11,12]. The most frequent site of perforation was the sigmoid colon, as in other studies^[10,13,18,23,24]. This may be explained by its anatomical characteristics of frequent redundancy or narrowing from diverticular disease or adhesions after previous pelvic operations^[25].

In conclusion, although the rate of perforation during colonoscopy is low, it is a serious complication and its early recognition and treatment are essential to optimize outcome. Surgery is mandatory in all patients with generalized peritoneal irritation. Early operative intervention makes primary repair a good and safe option, with low morbidity and mortality, unless there is a colonic pathology that necessitates resection. Selected patients with localized peritoneal irritation can be managed nonoperatively.

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RAPID COMMUNICATION

Liver microcirculation after hepatic artery embolization with degradable starch microspheres *in vivo*

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Abstract

AIM: To observe the dynamic changes of liver microcirculation *in vivo* after arterial embolization with degradable starch microspheres (DSM).

METHODS: DSM were injected into the proper hepatic artery through a silastic tube inserted retrogradely in gastroduodenal artery (GDA) of SD rats. Fluorescent microscopy was used to evaluate the dynamic changes of blood flow through the terminal portal venules (TPVs), sinusoids and terminal hepatic venules (THVs). The movements of DSM debris were also recorded. Six hours after injection of DSM, percentages of THVs with completely stagnant blood flow were recorded.

RESULTS: Two phases of blood flow change were recorded. In phase one: after intra-arterial injection of DSM, slow or stagnant blood flow was immediately recorded in TPVs, sinusoids and THVs. This change was reversible, and blood flow resumed completely. In phase two: after phase one, blood flow in TPVs changed again and three patterns of blood flow were recorded. Six hours after DSM injection, 36.9% ± 9.2% of THVs were found with completely stagnant blood flow.

CONCLUSION: DSM can stop the microcirculatory blood flow in some areas of liver parenchyma. Liver parenchyma supplied by arteries with larger A-P shunt is considered at a higher risk of total microcirculatory blood stagnation after injection of DSM through hepatic artery.

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Key words: Degradable starch microsphere; Hepatic microcirculation; Hepatic arteries; Fluorescence; Transarterial chemoembolization

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after hepatic artery embolization with degradable starch microspheres *in vivo*. *World J Gastroenterol* 2006; 12(26): 4214-4218

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INTRODUCTION

It has been accepted that transarterial chemoembolization (TACE) is an effective method to treat the unresectable hepatocellular carcinoma (HCC). With the characteristics of long retention time in the tumor tissue, iodinated poppyseed oil (Lipiodol) has been frequently used as the embolization material in clinical practice^[1-4]. Animal experiments demonstrated that after injection of iodized oil into the hepatic artery, small Lipiodol drops could be found in the terminal portal venules (TPVs), which was assumed passing through the pathway of arterial-portal anastomosis such as the peribiliary plexus^[5-7]. When Lipiodol drops flow into the sinusoids, they can severely occlude the blood flow, cause the stagnation of local microcirculation, and further lead to ischemic liver parenchyma injury^[8,9]. Super-selective technique with microcatheter and guidewires has been considered as a safe and effective way to treat HCC. Under some conditions in which liver function is severely damaged or blood supply of HCC is so complex that it is impossible to super-select the tumor feeding arteries, TACE is developed. Degradable starch microspheres (DSM), a temporary artery embolizer has been increasingly used as an alternative to Lipiodol in some particular situations^[10-12]. It has also been suggested that, when the tumor feeding artery cannot be super-selected by microcatheter and guidewire, one-shot injection of DSM before TACE can be regarded as a practical method to protect the tumor free liver tissue from the injury caused by Lipiodol inflow following TAE. To fully understand its effects on liver microcirculation, we injected DSM through proper hepatic artery of rats, and the dynamic changes of liver microcirculation were evaluated by *in vivo* fluorescent microscopic observations.

MATERIALS AND METHODS

Animal model

Ten Sprague-Dawley rats weighing 300 to 450 g were used in compliance with the regulations and the Guide for the Care and Use of Laboratory Animals. The animals were

Table 1 Blood flow in phase one in rats (min)

No.	Start of blood flow stagnation	Time of complete recovery of blood flow
1	1.1	10.9
2	1.4	13.1
3	2.6	15
4	1	14.9
5	1.6	9.82
6	2.3	11.5
7	2.8	13.2
8	3	14.5
9	2.8	14.3
10	1.6	9.47
mean \pm SD	2.0 \pm 0.8	12.7 \pm 2.1

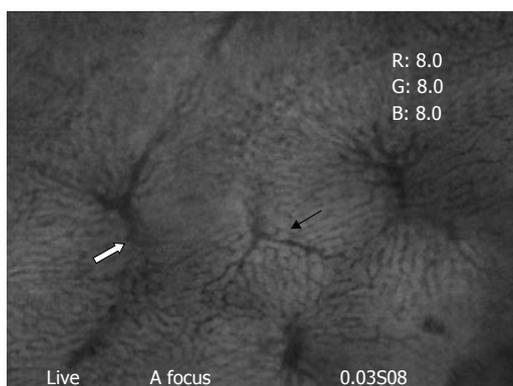


Figure 1 Normal microcirculation of the rat liver. Arrow: TPV, Opened arrow: THV.

fed with standard food pellets and tap water *ad libitum*. They were deprived of food but obtained free access to water for 12 h before the experiments. Anesthesia was performed by intra-peritoneal injection of 50 mg/kg sodium pentobarbital. The left femoral vein was cannulated with a 1F silastic tube (Natsume Corp. Tokyo, Japan) for additional anesthesia and liquid transfusion during the procedure. After a midline abdominal incision, the liver was carefully retracted to expose the gastroduodenal artery (GDA), which was catheterized by another 1F silastic tube (Natsume Corp., Tokyo, Japan) with its tip placed before the bifurcation that leads to the proper hepatic artery. The left lobe of liver was gently exteriorized and positioned over the window of the microscope stage. The liver parenchyma was covered with a small piece of plastic wrap; its surface was constantly irrigated with Ringer's solution at the body temperature.

Fluorescence microscopy

The exteriorized left liver lobe was transilluminated with monochromatic light generated by a prism monochromator equipped with a xenon lamp. Microscopic images of the microvasculatures were obtained with objective lenses (magnification, $\times 10$, $\times 20$) and an ocular lens (magnification, $\times 10$). DSM (Yakult Honsha Co., Ltd., Tokyo, Japan) 12 mg in 0.2 mL was prepared in a 1 mL syringe and the solution was made uniform before injection. After infusion of 1 g/L fluorescent sodium 0.1 mL into the cannulated GDA, DSM was injected gently in one minute. The *in vivo*

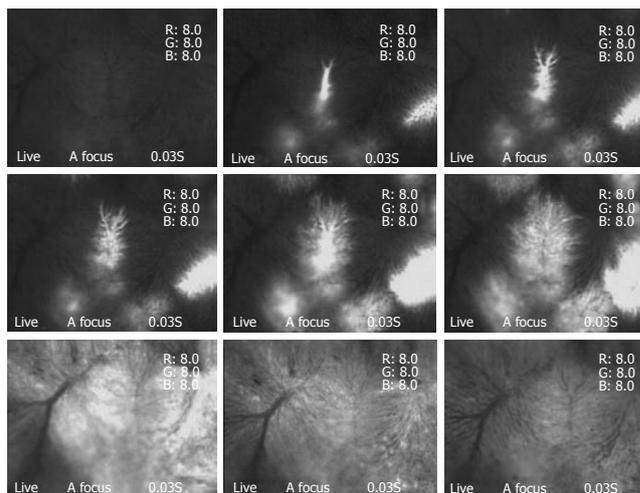


Figure 2 After infusion of fluorescent sodium from the GDA to proper hepatic artery, blood flow from TPVs to THVs through sinusoids can be clearly visualized.

microscopic images of the following procedure were recorded on videotapes.

In vivo evaluation

For each rat, areas with best visualization were selected for evaluation. Six hours later, 1 g/L fluorescent sodium 0.1 mL was infused through the cannulated left femoral vein to check the whole surface of liver lobe for a complete confirmation of the liver microcirculation. One hundred THVs were randomly selected during the horizontal and vertical movements of the microscope. THVs with completely stopped blood flow were statistically counted.

Statistical analysis

Data analysis was performed employing the Statistical Package for the Social Sciences Version 12.0 for Windows (SPSS[®] Inc., Chicago IL, USA). Results of the descriptive statistical analysis were presented as mean \pm SD.

RESULTS

Clear images of the liver microcirculation (TPVs, sinusoids, and THVs) could be seen under *in vivo* fluorescent microscope (Figure 1). Blood flow from one TPV was drained through the sinusoids into several THVs; similarly, one particular THV provided venular drainage for several TPVs. Hepatic arterioles, the other afferent vessels in the liver, usually could not be visualized (Figure 2).

Blood flow in TPVs after DSM injection

Blood flow through TPVs demonstrated an immediate response after DSM injection. The speed of blood flow dropped dramatically at once. In 2.0 ± 0.8 min (Max 3 min), the blood flow in the observed area completely stopped. After that, blood flow through TPVs resumed gradually; 12.7 ± 2.1 min (Max 15 min) after injection of the DSM, blood flow through TPVs completely recovered (Table 1). No evidence of DSM or its disaggregated debris could be recorded within this time interval. For convenient explanation, we named this period as "phase one", and the

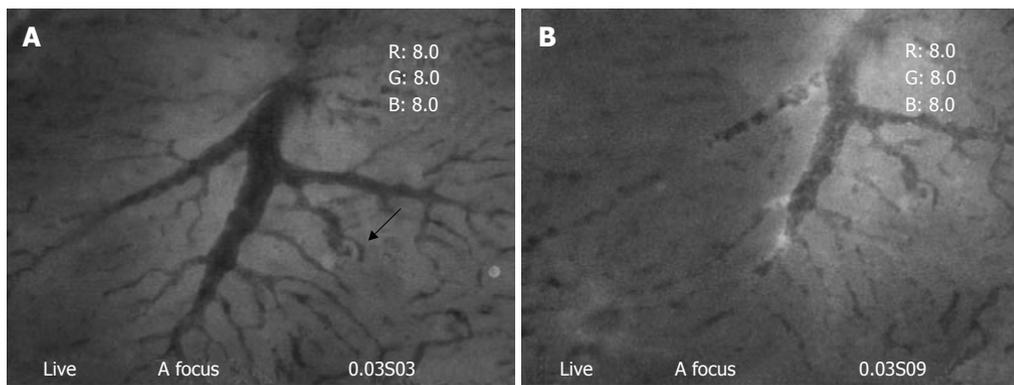


Figure 3 A: Small debris can enter sinusoids through TPV (arrow); B: The same TPV as image 3, debris of DSM continuously enter sinusoids through TPV, blood flow in TPV is intermittent but not stagnant.

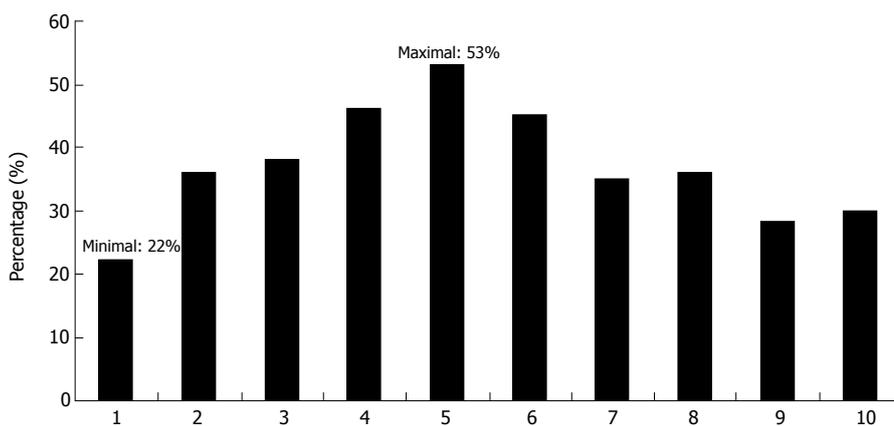


Figure 4 Percentage of the completely stagnant THVs 6 h after DSM injection in 10 rats. The minimal percentage is 22%, and the maximal is 53%.

blood flow changes afterward as “phase two”. In phase two, three different types of blood flow changes in TPVs could be recorded.

Type one: The speed of blood flow slowed down again, and then completely stopped. This phenomenon could be found as early as 25 min after DSM injection and last for the whole procedure. The TPVs could never resume their blood flow during the observational period. DSM or its debris could be hardly found in TPVs in this type.

Type two: The speed of blood flow in TPVs decreased at different level, either slightly striking or some times intermittent, however the stagnation was not recorded during the whole observation time. Numerous small pieces of debris with irregular shapes could be found to flow through and drain into the distal sinusoids (Figure 3A and B).

Type three: In particular areas, TPVs kept a constant flow after phase one. The blood flow did not change during the whole procedure. DSM or its debris could not be recorded in these TPVs.

Blood flow in sinusoids and THVs

The blood flow through sinusoids and THVs followed the changes of TPVs in phase one. They also demonstrated a dramatic decrease of blood flow speed after DSM injection. Some even completely stopped. Nevertheless, it could be fully recovered.

During the following period, three kinds of blood flow, similar to that of the TPVs could also be recorded in sinusoids and THVs, ie, completely stagnant blood flow, intermittent and slow flow, normal flow.

DSM debris in the hepatic microvasculature

Twenty minutes (the earliest time) after injection of DSM, small pieces of debris could be found in some sinusoids. Some of the debris, with a relatively small size, could directly pass through the sinusoids and flow into THVs. Some debris, with larger size, could occlude the corresponding sinusoids. This occlusion was temporary; recanalization could be achieved by opening of collaterals or further distal movement of the disaggregated debris. The number of DSM debris reached a peak value 1 h after DSM injection. No DSM particles with the original size and shape could be found in sinusoids and THVs. Numerous disaggregated debris with small diameter entered the THVs.

Hepatic microcirculation after DSM injection

Six hours after DSM injection, the brightness of the liver surface was not uniform after infusion of fluorescent sodium through the femoral vein, suggesting the heterogeneous nature of the liver blood flow. Areas with completely stagnant blood flow in TPVs, THVs and sinusoids could be found sparsely distributed among the areas with normal or sluggish blood flow. Approximately $36.9\% \pm 9.2\%$ of randomly selected THVs were found with completely stagnant blood flow (Figure 4).

DISCUSSION

DSM is a kind of embolization material that can temporarily occlude the vessels. The degradation of

DSM is considered to be caused by a combination of the chemical effects of amylase and the striking force of the vortexial arterial flow. Hepatic arterial perfusion is essential for an optimal sinusoidal function because it maintains transsinusoidal pressure^[13]. After intra-artery injection of DSM, immediately slow down or even stop of the blood flow in TPVs, sinusoids, and THVs could be found. It is considered that a sudden reduction of arterial blood flow is caused by numerous DSM casts embolization. The blocked blood flow could soon be resumed due to a compensatory increase of portal blood flow as a buffer response. This can explain the phenomenon of phase one which happened after the DSM injection. After a complete recovery of blood flow in phase one, presinusoidal A-P shunt^[14-16] should be the reason for the appearance of three types of blood flow in phase two. We have found in our previous study that after intra-arterial injection of DSM, various sizes of DSM casts are formed inside the arterioles which can block the arterial blood flow. The proximal end of DSM casts will disaggregate under the pumping force of vortexial arterial blood flow. Debris with different sizes will be discharged at the proximal end and further occlude the branch of the original artery. We presume that A-P shunts have various size, some larger debris of the disaggregated DSM can pass through larger A-P shunts and reach the portal side that is proximal to TPVs. Debris accumulated at the portal side form a number of emboli. These emboli, if big enough, will completely shut the portal blood flow to distal TPVs and be harder to disaggregate because the pumping force of portal blood stream is much weaker than that of the arterial one. This means, at certain areas of liver parenchyma, both arterial and portal blood flows are stopped by DSM casts and the debris emboli. Few debris could be found in the distal TPVs because the proximal portion of TPVs was completely occluded by the debris emboli. This explains the type one phenomenon. For some small A-P shunt, only small-sized debris can pass through; and these smaller debris could partially occlude portal branch and was easily to be pushed distally. Some could reach TPVs and flow through sinusoids to THVs. This caused type two phenomenon. As for the type three phenomenon, it is considered that no debris of DSM entered portal site through A-P shunt.

After entering sinusoids, debris could occlude sinusoids. For a single sinusoid, the blood flow can be resumed either by further disaggregation of the debris or by opening of the small collaterals. Small DSM debris, when passing through sinusoid, will flow freely into THVs. Six hours after injection of DSM, we found 22%-53% (mean: 36.9% \pm 9.2%) of THVs with totally stagnant blood flow. That means all sinusoids draining blood to these THVs had been stagnant in blood flow. The corresponding liver parenchyma received no fresh blood supply during this time. The cause of stagnant blood flow in sinusoids surrounding THVs is presumed at the presinusoidal level. We assume that the occlusion site was the presinusoidal portal vein with relatively larger diameter. After arterial embolization by DSM casts, the more bigger debris of DSM entered this portion through larger A-P shunt and accumulation of these debris formed intravascular

emboli. Weak pumping force of portal blood flow could not disaggregate these emboli, and the amylase could take effects more slowly because little fresh blood flow could reach those emboli. With all those factors, the emboli could maintain stable during a fairly long period of time. Thus, a simultaneous blockade of the arterial and portal blood flow could lead to a completely stagnant blood flow in distal sinusoids. Because the amylase in blood flow will chemically disaggregate the DSM and its debris, whether the TPVs, sinusoids and THVs can resume their blood flow later needs to be further studied.

It is preliminarily confirmed in this study that DSM, with its degradation products, can enter portal vein through hepatic arterial injection. It can completely stop the microcirculatory blood flow in some areas of liver parenchyma. A-P shunt is considered to be a determining factor during the procedure. Liver parenchyma supplied by arteries with larger A-P shunt is presumed to have higher risk of total microcirculatory blood stagnation after injection of DSM through hepatic artery. Whether the use of DSM can provide protective effects during TACE awaits further evaluation.

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Mutations in surface and polymerase gene of chronic hepatitis B patients with coexisting HBsAg and anti-HBs

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Abstract

AIM: To investigate the clinical significance and presence of mutations in the surface (S) and overlapping polymerase gene of hepatitis B patients with coexisting HBsAg and anti-HBs.

METHODS: Twenty-three patients with chronic hepatitis B were studied. Of the 23 patients, 11 were both positive for hepatitis B virus (HBV) surface antigen (HBsAg) and antibody to HBV surface antigen (anti-HBs), 12 were negative for anti-HBs while positive for HBsAg. DNA was extracted from 200 μ L serum of the patients. Nucleotide of the surface and overlapping polymerase gene from HBV-infected patients was amplified by PCR, and the PCR products were sequenced.

RESULTS: Forty-one mutations were found within the surface gene protein of HBV in 15 patients (10 with coexisting HBsAg and anti-HBs). Six (14.6%) out of 41 mutations were located at " α " determinant region in 5 patients (4 positive for HBsAg and anti-HBs). Eleven mutations (26.8%) occurred in the downstream or upstream of " α " determinant region. Lamivudine (LMV)-selected mutations were found in three patients who developed anti-HBs, which occurred in amino acid positions (196, 198, 199) of the surface protein and in YMDD motif (M204I/V) of the polymerase protein simultaneously. Presence of these mutations did not relate to changes in ALT and HBV DNA levels.

CONCLUSION: Besides mutations in the " α " determinant region, mutations at downstream or upstream of the " α " determinant region may contribute to the development of anti-HBs. These mutations do not block the replicating competency of HBV in the presence of high titer of anti-HBs.

INTRODUCTION

Hepatitis B virus (HBV) infection leads to a wide spectrum of liver diseases, including acute self-limited infection, asymptomatic carrier state, fulminant and chronic hepatitis, which could result in life-threatening sequelae, such as liver cirrhosis and hepatocellular carcinoma^[1]. In acute self-limited infection, clearance of HBV is associated with seroconversion from HBsAg to anti-HBs due to the coordination of humoral and cellular immune response^[2,3]. However, this condition is very rare in chronic infection patients, especially in patients infected at birth, partly because of inadequate humoral and cellular immunity of the host^[4]. Furthermore, the prevalence of HBV mutations that could escape from humoral and cellular immunity may result in persistent virus infection.

The S gene of HBV has three open reading frames (ORF), including preS1, preS2 and S region. The surface gene contains a neutralizing epitope named " α " determinant region located at the condon positions 124-147. Mutations in this region could alter the antigenicity of HBsAg, causing failure of anti-HBs to neutralize HBsAg and escaping from the host's immune system, resulting in active viral replication and liver disease^[5,6]. It is reported that mutations of some epitopes located at downstream of the " α " determinant region may also affect the neutralization domain^[7]. The surface gene overlaps with the catalytic domains of polymerase^[7]. Thus, mutations in the surface gene have an effect on the polymerase gene while the polymerase gene mutations also impact the surface gene^[8,9].

The present study was to analyze the prevalence of mutations in the surface and polymerase gene of HBV in patients with coexisting anti-HBs and HBsAg.

MATERIALS AND METHODS

Patients

Sera were obtained from 23 Chinese patients with chronic HBV infection. Presence of HBsAg, anti-HBs, hepatitis e antigen (HBeAg), antibody to HBeAg (anti-HBeAg) was detected by commercially available kits according to the instructions. All patients were positive for HBsAg. Of the 23 patients, 11 were positive for anti-HBs (No. 1 to 11) and 20 were positive for HBeAg. Fifteen out of the 23 patients had elevated alanine aminotransferases (ALT) levels. Virological and biochemical parameters of 8 patients positive for anti-HBs and HBsAg at the time of HBV sequence analysis were analyzed. No patients had a history of HBV vaccination or hyper immune globulin therapy. Three patients positive for HBsAg and anti-HBs had a history of lamivudine (LMV) therapy for more than 1 year. All patients were negative for antibody to hepatitis C virus. Sera were stored at -20°C for DNA extraction.

Primer synthesis

For polymerase chain reaction (PCR), primers were synthesized according to the published sequences. Sequences of the primers for amplifying the surface gene of HBV are as follows: HBV S1: 5'-TTACAGGCGGGGTTTTC-3' (nt 197, sense); HBV S2: 5'-AAGGGACTCAAGATG-3' (nt 789, anti-sense). Primers of HBV P1, 5'-GTATTCATCCATCCATCC-3' (nt 599, sense) and HBV P2, 5'-CAAGGCAGGATAGCCACATT-3' (nt 1033, anti-sense) were used for amplification of polymerase gene of HBV.

PCR amplification

DNA was extracted from 200 µL serum using a blood DNA kit (Omega, USA). Two microlitres of DNA template and 1 µL of each of the primers, 2 µL of 10 × dNTP and 0.5 U of Taq DNA polymerase (Promega, US) were used in a volume of 50 µL for PCR. After denaturation at 94°C for 5 min, the reaction for amplification of the surface gene with primers HBV S1 and HBV S2 was carried out at 94°C for 30 s, at 56°C for 1 min, and at 72°C for 1 min for 35 cycles, and a final extension at 72°C for 10 min. The reaction with primers HBV P1 and HBV P2 for amplifying the polymerase gene was performed at 94°C for 30 s, at 56°C for 30 min, at 72°C for 1 min for 35 cycles, and a final extension at 72°C for 10 min.

Sequencing of PCR products

The PCR products were purified by centrifugation. Direct sequence of the gene was determined using Taq Dye-Deoxy terminator sequencing kits. Sequencing reactions were analyzed on an automated DNA sequencer (model 377, ABI100, Applied Biosystem). Deduced amino acid sequences were compared with the reported consensus sequence of genotype C, subtype adw, HBV clone (PAK66, PIWK146). Mutations were determined as sequence difference from the consensus sequence.

The nucleotide sequence data presented in this paper could be found in the DDBJ/EMBL/GeneBank nucleotide sequence databases with the access numbers AB014381,

Table 1 Clinical data of two groups of patients

Clinical factors	Groups		P
	Positive anti-HBs	Negative anti-HBs	
Age in years	40.3 ± 13.2	42.5 ± 14.7	0.7672
Sex, M/F	5/6	7/5	0.6531
Patients with HbsAg, n (%)	11 (100)	12 (100)	
Patients with HbeAg, n (%)	9 (81.8)	11 (91.7)	
Patients with anti-HBe, n (%)	1 (9.1)	0 (0)	
ALT in IU/L	65.9 ± 30.5	98.9 ± 42.0	0.6541
HBV-DNA (log)	7.0 ± 1.60	6.87 ± 0.9	0.5263
Number of amino acid residues			
mutations in S gene, n (%)	34/41 (82.9)	7/41 (17.1)	

Fisher's exact test was used for the categorized data; two-tailed Student's *t* test was used for ALT levels, age and HBV DNA levels (log, copies mL⁻¹).

AB033554, AY812744, AY812743, AY800249, AY123424, AF100309.

Statistical analysis

Two-tailed Student's *t* test was used to assess the difference in ALT levels, age, HBV DNA levels between the two groups of patients. Fisher's exact test was used for the analysis of difference in mutations between the two groups. *P* < 0.05 was considered statistically significant.

RESULTS

Comparison of the clinical features between the two groups of patients is shown in Table 1. There was no significant difference in ALT levels, age, HBV DNA levels between the two groups (*P* > 0.05). The relevant biochemical and virological parameters of 8 patients (No.1 to 6, No.8 and 10) are shown in Table 2.

Nucleotide and deduced amino acid sequences of surface region and polymerase gene of HBV were performed in 23 patients. Comparison with the published HBV sequence showed that 21 (91.3%) out of 23 patients were infected with genotype C, 1 with genotype B and 1 with genotype D.15 (65.2%). Of the 23 patients who developed amino acid mutations in the surface gene protein, 10 were positive for anti-HBs and 5 were negative for anti-HBs. Mutations at the "α" determinant region were observed in 5 patients (5/15, 33.3%) (Figure 1). Forty-one mutations were found at 27 amino acid positions within the surface gene of HBV, and 34 mutations (82.9%, 34/41) were presented in the patients with coexisting HBsAg and anti-HBs. Six (14.6%) out of 41 mutations were located at the "α" determinant region, and 4 mutations were presented in the first loop (positions 124-137), the others were in the second loop (positions 139-147, S143T, G145R). Six mutations at amino acid residues 40 (N40S) and 47 (T47V, T47K, T47R) coincident with HLA class I-restricted (CTL) epitope^[10] were observed in 5 patients, 11 mutations (26.8%) occurred in 6 patients within the major hydrophilic regions of upstream and downstream of the "α" determinant region (amino acid positions 99-169), 6 mutations at 3 amino acid positions (196, 198 and 199) associated with LMV-selected

Table 2 Virological and biochemical follow-up data of 8 patients

No.	Sex	Age	2001				2002				2003				2004			
			ALT	HBVDNA	a-HBs	HBeAg	ALT	HBVDNA	a-HBs	HBeAg	ALT	HBVDNA	a-HBs	HBeAg	ALT	HBVDNA	a-HBs	HBeAg
1	M	57	22	0	-	-	20	0	-	-	76	8.63	-	+	87	6.38	+	+
2	M	36	48	9.38	-	+	82	8.53	-	+	21	4.04	-	+	97	3.78	+	+
3	F	65	25	4.04	+	-	58	5.04	+	-	55	6.76	+	-	55	5.08	+	-
4	M	45	45	6.86	+	+	80	6.61	+	+	59	5.57	-	+	93	6.99	+	+
5	M	55	156	7.32	+	+	116	7.11	+	+	112	7.75	+	+	149	5.80	+	+
6	F	30	19	6.91	+	+	33	7.70	+	+	20	8.86	-	+	15	9.08	+	+
8	M	38	18	0	+	-	19	0	+	-	20	0	-	-	21	4.43	+	-
10	M	72	20	5.18	+	+	25	7.04	+	+	18	6.26	-	+	48	4.15	+	+

* Positive result; * Negative result; ALT: alanine aminotransferase; HBV DNA: HBV DNA levels (log, copies ml⁻¹).

Table 3 Mutations of HBV in polymerase and HBsAg protein

Mutant in patients (n)	Position with HBsAg protein sequence change				Position with polymerase protein sequence change			
	145	196	198	199	173	180	204	223
Wild type	G	W	M	W	V	L	M	S
1	-	L	-	-	-	-	I	A
3	-	L	-	-	-	-	I	-
10	-	F	-	C	L	M	V	-
11	R	-	-	-	-	-	-	A
13	-	L	-	-	-	M	-	-

mutation were observed in 5 patients.

Because the S gene overlaps with the major catalytic domain of the polymerase gene, the mutations near the YMDD motif of the polymerase gene were studied. Eight mutations within amino acid residues 518-569 of the polymerase gene were observed at 4 positions (V173L, L180M, M204I/V, S223A) in 5 patients. Three patients who received long term LMV therapy and developed anti-HBs at the time of sequencing, had YMDD mutations (M204I/V) in polymerase gene and the S gene mutations at amino acid positions 196, 198 and 199 (Table 3).

Five out of 15 (33.3%) patients who had amino acid mutations did not develop anti-HBs, while T131N, L162Q, W196L mutations in the S gene and L180M mutation in polymerase gene were simultaneously observed in only one of these patients.

DISCUSSION

HBV is the most common etiologic agent of chronic and often fetal liver diseases world wide. HBV variants present during natural infection or anti-virus therapy, and contribute to disease persistence. The S gene of HBV is crucial for binding and infectivity, and "α" determinant of the surface gene may form a target of humoral neutralizing antibody. Mutations in the region affect the binding of anti-HBs to corresponding HBsAg^[5], produce escape from the neutralizing antibody, result in persistent infection and replication of HBV, even the relatively high titer anti-HBs develops.

In the present study, 15 patients had amino acid

mutations in the surface gene of HBV, and 10 of them were positive for anti-HBs. Forty-one mutations were found in 23 patients, and 34 (82.9%) mutations were presented in the patients with coexisting HBsAg and anti-HBs. only 6 mutations within the "α" determinant region were observed in 5 patients (4 for anti-HBs positive). Passive or active immune therapy may develop the escape mutation^[11,12], which has a point mutation from guanosine to adenosine at nucleotide 587 (condon 145, G145R). Mutation of G145R, however, was only seen in 1 patient in our study, and it may be the reason why no patient in our cohort received active or passive hepatitis B immunization. The data suggest that mutation within the "α" determinant region may play an important role in the presence of anti-HBs. A recent study showed that mutation in the "α" determinant region contributes to the most therapy failure, but there are still some therapy failures associated with mutations in the major hydrophilic region of the surface gene located at downstream or upstream of the "α" determinant (positions 99-169)^[13]. In the present study, 26.8% mutations were observed in the above mention region, suggesting that these mutations also change the antigenicity of HBsAg and contribute to the development of anti-HBs. One patient who had amino acid mutation (T131N) in the "α" determinant region did not develop anti-HBs. It may be due to the relatively lower sensitive assays because it was reported that anti-HBs complexed with HBsAg could be detected in nearly all patients with chronic hepatitis B when tested by a highly sensitive immunoassay^[14].

Lamivudine-selected mutations in the S gene of HBV have been demonstrated by sequencing HBV isolated from the serum of patients treated with long-term LMV^[15,16]. In addition, LMV-selected mutations within the HBsAg protein downstream of the "α" determinant (I195M, W196S and M198I) lead to a decrease in the antigenicity of the protein and binding to the anti-HBs antibodies, therefore poorly inhibiting their interaction with wild-type HBsAg^[17]. In our study, the change of methionine to isoleucine (rtM204I) or valine (rtM204V) was found in the YMDD motif of the polymerase gene protein in three patients who received long-term LMV therapy. These patients also had W196L/W196F or W199C mutations within the surface gene protein of HBV simultaneously.

The surface gene of HBV also includes the putative

No.	14 V	25 I	40 N	47 T (V)	64 S	76 C	85 F	89 L	95 L	99 D	101 Q/H	110 L (I)	113 T (S)	117 S	122 K (R)	125 T (M)	131 T (N)	134 F (Y)	143 S	145 G	159 A (G)	162 L (Q)	175 L	196 L	198 W M
1	G	-	S	V	C	Y	C	I	-	-	I	S	-	-	-	-	-	T	-	-	-	-	L	-	
2	-	-	-	-	-	-	-	-	-	G	-	-	T	-	-	-	-	-	-	-	-	-	-	-	
3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	L	-	
4	-	-	-	-	A	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	T	
5	-	-	-	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
7	-	-	-	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
8	-	-	-	V	-	-	-	-	-	-	I	S	-	R	M	-	Y	-	-	G	-	-	-	-	
9	-	F	-	-	-	-	-	-	-	-	R	-	-	-	M	-	-	-	-	-	-	S	-	-	
10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	F	-	
11	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	-	R	-	-	-	-	-	
12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N	-	-	-	-	Q	-	L	-	
13	-	-	-	-	-	-	-	-	W	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
14	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
15	-	-	-	-	-	W	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
16	-	-	-	R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
17-23	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	

Figure 1 Amino acid mutations in the surface gene of HBV. Positions of mutation in deduced amino acid residues are indicated by vertical line below the surface protein of HBV. The consensus sequences of A, B and D different from those of genotype C are listed in parentheses. Dashes mean residues identical to these reference residues. Patients 1 to 11 were positive for HBsAg and anti-HBs, the others were negative for anti-HBs.

HLA class I-restricted cytotoxic T lymphocyte (CTL) epitopes^[2]. Because the putative CTL epitope mutations result in epitope inactivation and T cell receptor antagonism^[18], mutant virus could evade cellular immunity and lead to persistent infection^[19,20]. In the patients studied here, 6 mutations at amino acid residues 40 and 47 were observed in 1 and 5 patients, respectively, and 5 of them were positive for anti-HBs. These patients had relatively high HBV DNA level. Three of them had elevated ALT levels. This result is similar to the report from Taiwan, which revealed a high frequency of mutations at amino acid positions 40 and 47 of the surface gene in patients with chronic hepatitis B, suggesting that these mutations change CTL recognition and contribute to chronic infection in some patients^[10].

In fact, patients positive for anti-HBs have more amino acid mutations, especially mutations in the crucial region of the surface gene associated significantly with the presence of anti-HBs. But presence of these mutations is not related to clinical features, ALT levels, HBV DNA levels and the presence of HBeAg, suggesting that these mutations may not alter the replicating competency of HBV although highly titer of anti-HBs develops. It is possible that these mutants may secrete into the serum via trans-complementation of intact protein in hepatocytes. The biochemical and virological follow-up parameters of 8 patients (No.1 to 6, No.8 and 10) showed that only 2 patients had persistent anti-HBs positive condition during the whole 4 year follow-up period, indicating that anti-HBs can be detected in chronic hepatitis B patients.

In conclusion, the presence of mutations in the “α” determinant of surface gene is not high in patients with coexisting anti-HBs and HBsAg. The mutations at the major hydrophilic region of the surface gene contribute to the development of anti-HBs in these patients and produce escape from the neutralizing antibody, and lead to

persistent infection. Long-term LMV therapy could induce YMDD mutation in the polymerase gene and surface gene of HBV.

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RAPID COMMUNICATION

Pancreatic encephalopathy and Wernicke encephalopathy in association with acute pancreatitis: A clinical study

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Abstract

AIM: To investigate clinical characteristics and therapy of pancreatic encephalopathy (PE) and Wernicke encephalopathy (WE).

METHODS: In a retrospective study of 596 patients with acute pancreatitis (AP), patients with PE were compared to those with WE in regards to history, clinical manifestation, diagnosis, treatment and outcome.

RESULTS: There were 93 patients with severe acute pancreatitis (SAP). Encephalopathies were discovered in 10 patients (1.7%). Six patients with PE all developed in SAP (6.5%), and three of them died (3% of SAP, 50% of PE). Four patients with WE developed in AP (0.7%), and two of them died (0.3% of AP, 50% of WE). Two patients with WE were treated with parenteral thiamine and survived. Global confusions were seen in all patients with encephalopathy. Ocular abnormalities were found. Conjugate gaze palsies were seen in 1 of 6 (16.7%) patients with PE. Of 4 patients with WE, one (25%) had conjugate gaze palsies, two (50%) had horizontal nystagmus, three (75%) had diplopia, and one (25%) had myosis. Ataxia was not seen in all patients. None of patients with WE presented with the classic clinical triad. CSF examinations for 2 patients with WE showed lightly-increased proteins and glucose. CT and MRI of the brain had no evidence of characteristic abnormalities.

CONCLUSION: PE occurs in early or reiteration stage of SAP, and WE in restoration stage of SAP/AP. Ocular abnormalities are the hallmarks of WE, and horizontal nystagmus is common. It is difficult to diagnose earlier an encephalopathy as PE or WE, as well as differentiate one from the other. Long fasting, hyperemesis and total parenteral nutrition (TPN) without thiamine are main causes of thiamine deficiency in the course of pancreatitis.

INTRODUCTION

Pancreatic encephalopathy (PE) is an uncommon complication of acute pancreatitis (AP). PE, which is one of multiple organ dysfunction syndrome (MODS), generally occurs in early stage of severe acute pancreatitis (SAP) and has a high mortality of 57%^[1]. But in the last or restoration stage of AP, neurological complications are mostly Wernicke encephalopathy (WE) which results from long fasting, hyperemesis and total parenteral nutrition (TPN) without thiamine (vitamin B1, Vit B1). A large dose of Vit B1 is certainly effective for WE. However, it is difficult to diagnose earlier an encephalopathy as PE or WE, as well as differentiate one from the other. Recent studies^[2] have shown that WE is poorly recognized by clinicians, even when features of the classic triad of symptoms are evident. Recognition of the progressive nature of the disease is critical because the mortality rate is as high as 10% to 20%^[3-5], and treatment may correct all abnormalities. This study demonstrates many of the common clinical characteristics of WE and the diagnostic dilemma physicians encounter when confronted with WE. By reviewing our clinical experience, we can learn many lessons which are beneficial for physicians, not just those who care for patients with PE.

MATERIALS AND METHODS

Patients and methods

A retrospective study was conducted on 596 patients with AP hospitalized at China PLA General Hospital over a 10-year period from Jun 1993 to Dec 2003. There were 93 patients with SAP. A chart was reviewed containing the following demographic and clinical data: age, sex, clinical

Table 1 Manifestation and outcome of patients with encephalopathy

No.	Sex	Age (yr)	Primary disease	Manifestation of encephalopathy	Diagnosis	Treatment/Outcome
1	M	29	SAP with ARDS.	4 th d of onset, restlessness, haziness, delirium. Pathological sign negative.	PE	Diazepam, haloperidol. Recovery
2	F	51	SAP	At onset, haziness, delirium.	PE	Recovery
3	M	41	SAP with ALI.	4 th d of onset, restlessness, sleepiness, haziness.	PE	Recovery
4	M	34	SAP with pseudocyst bleeding	SAP for 3 mo, 3 rd d after pseudocyst operation, hallucination, delirium, conjugate gaze palsies, coma, suspected Kernig sign. Diffused pancreatic necroses.	PE	Death
5	F	42	SAP with ALI and shock	9 th d of onset, delirium, unconsciousness.	PE	Death
6	M	31	SAP with ARF and ARDS.	33 rd d after onset, restlessness, hebetude, unconsciousness, delirium.	PE	Death
7	F	37	Recovery Phase of AP	Protracted vomiting. No supplement of VitB ₁ . 36 th d after onset, diplopia, tinnitus, apathy, dizziness, horizontal nystagmus. CSF negative, MRI negative.	WE	VitB ₁ , B ₁₂ im. Recovery after 4 d
8	M	48	Recovery Phase of SAP, with ALI and pseudocyst	45 th d of onset, diplopia, sleepiness, haziness, horizontal nystagmus, spatial disorientation, decreased tendon reflex.	WE. Once suspected PE	Fasting for 51 d, no VitB ₁ in TPN for 44 d. Recovery after 4-day's administration of VitB ₁
9	M	37	Acute recurrent pancreatitis with pseudocyst	Long fasting, no supplement of VitB ₁ . Nausea, vomiting, dizziness, hypomnesia, alalia, diplopia, amentia, coma. Conjugate gaze palsies, active tendon reflex, ankle clonus positive. CSF: total cells 134, WBC 2; glucose, protein increased lightly. MRI: suspected focus of brain stem.	WE. Once suspected PE and encephalitis	Dexamethasone ineffective. Administration of VitB ₁ , 400 mg/d. Death
10	F	40	Recovery phase of AP	42 nd d after onset, vomiting, dizziness, alalia, trance, amentia, sleeplessness, hyperspasmia, coma. Myosis, decreased tendon reflex. Pathological signs negative. CSF: protein positive; total cells 180, WBC 0; glucose, protein increased lightly.	WE. Once suspected viral encephalitis	Dexamethasone, acyclovir ineffective. Respirator. Death

ALI: acute lung injury; ARF: acute renal failure; ARDS: acute respiratory distress syndrome; CSF: cerebrospinal fluid; TPN: total parenteral nutrition.

signs, history, imaging, treatment, hospital course and outcome. Patients with PE were then compared to those with WE in regards to history, clinical manifestation, diagnosis, treatment, and outcome.

RESULTS

Encephalopathy was discovered in 10 patients (1.7%), including 6 males and 4 females with a mean age of 39.0 years (from 29 to 51 years old). Six patients with PE all developed from SAP (6.5%); three patients died (3% of SAP, 50% of PE). Four patients with WE developed from AP (0.7%); two patients died (0.3% of AP, 50% of WE). Two patients with WE were treated with parenteral Vit B1 and survived. PE occurred in early or reiteration stage of SAP, and WE in restoration stage of SAP/AP.

The clinical features were also reviewed in all patients (Table 1). Global confusions were seen in all patients with encephalopathy. Ocular abnormalities were also found. Conjugate gaze palsies were seen in 1 of 6 (16.7%) patients with PE. Of 4 patients with WE, 1 (25%) was seen conjugate gaze palsies, 2 (50%) horizontal nystagmus, 3 (75%) diplopia, and 1 (25%) myosis. Ataxia was not seen in any of the patients. None of the patients with WE presented with the classic clinical triad. CSF examination was performed on 1 patient with PE and showed negative, whereas it was done on 2 patients with WE and showed lightly-increased proteins and glucose. Of the 10 patients, 5 had CT scan of the brain and none had evidence of characteristic abnormalities. MRI was performed on 2 patients with WE and only 1 had some suspected changes of brain stem. Long fasting, protracted vomiting and TPN without Vit B1 supplement were main causes of Vit B1

deficiency, which crucially resulted in WE.

DISCUSSION

PE, first described by Lowell in 1923, refers to the abnormalities of mental status in patients with AP. Abnormalities of mental status, such as spatial disorientation, trance, agitation with delusion and hallucination, were defined as PE by Rothermich^[6,7]. In China, PE had a high mortality of 57%^[11], and main causes of death were shock, MODS, renal failure and ketoacidosis. At present pathogenesis of PE is unclear yet, and most scholars think that it is related to phospholipase A (PLA) activation, hypovolemia, multiple organ failure, electrolyte disturbance and cytokine effect in the course of AP^[8]. In recent years, it has been gradually known that PLA in pancreatitis is not only the primary factor causing pancreatic necrosis, but also crucial substance resulting in PE^[9]. Johnson *et al.*^[10] reported a male patient with AP. Cerebral fat embolism was established as the cause of his death. They thought that PE might be due to hypoxia secondary to pulmonary fat embolism, cerebral fat embolism, or the complicating syndromes of disseminated intravascular coagulation or hyperosmolality.

PLA2 damages structural phospholipid of brain cell membrane; platelet activating factor (PAF) increases intracerebral vascular permeability with brain edema and demyelination of grey and white matter. PE is primarily due to the demyelination of the cerebral grey and white matter caused by PLA2, which can induce increased vascular permeability. The intravascular osmotic pressure decreases and the brain becomes more vulnerable to transudation, and finally brain edema is resulted^[11].

With proper treatment, the recovery in patients aged

below 40 is uneventful. Those older than 60 especially those with a previous history of cerebral infarction may have some sequela^[11]. Ruggieri *et al*^[12] presented a patient of 43-year-old man who, after an acute episode of pancreatitis, experienced five relapses, with alternating focal signs. The patient had improved, but cognitive impairment persisted after a 7-year follow-up.

A report^[13] of Boon *et al* showed the usefulness of MRI in the diagnosis of this disorder. Patchy white matter signal abnormalities, resembling plaques seen in multiple sclerosis, might reflect the lesions that were found in the cerebral white matter of post-mortem confirmed patients.

Estrada *et al*^[7] conducted a prospective study on 17 patients with AP. PE was discovered in 6 patients (35%). A direct relationship was found to exist between the PE condition and an increase in CSF-lipase, and electroencephalographic changes were nonspecific. The encephalopathy did not affect the course of AP, and showed no relationship to type of treatments involved. Whereas the severity of AP was not related to the presence or absence of encephalopathy.

Our study revealed that PE occurred in early or reiteration stage of SAP and had a global confusional state for 2-7 d. Some patients had ocular abnormalities. There was no specific therapy for PE. Treatment of AP was the key to prevention and therapy of PE.

The clinical trial of Qian *et al*^[14] revealed that recombinant human growth hormone (rhGH) had a therapeutic effect for patients with early PE; rhGH combined with somatostatin might reduce occurrence of PE. However, the mechanism is unclear yet.

WE is an uncommon neurological disorder characterized by a triad of ocular abnormalities, ataxia, and global confusional state. In 1881, Carl Wernicke initially described punctate hemorrhages affecting grey matter around the third and fourth ventricle and aqueduct of Sylvius and designated it "polioencephalitis hemorrhagica superioris"^[15]. Experimental and clinical studies have demonstrated that WE results from a deficiency of thiamine (vit B1), an essential coenzyme in intermediate carbohydrate metabolism^[16,17].

The mortality rate ranges from 10% to 20%. At autopsy, patients may have pin-point hemorrhages in the mamillary bodies, hypothalamus, and paraventricular regions of the thalamus, around the aqueduct and beneath the floor of the fourth ventricle^[3-5]. A retrospective study of Ogershok *et al* showed similar pathological lesions at autopsy^[2].

Although WE is thought to be a disease that occurs primarily in the alcoholic population, Lindboe's autopsy study revealed 12 (23%) of 52 patients in nonalcoholic population^[4]. Some of the nonalcoholic conditions associated with this disorder include prolonged intravenous feeding, hyperemesis gravidarum, anorexia nervosa, refeeding after starvation, thyrotoxicosis, regional enteritis, malabsorption syndromes, hemodialysis, peritoneal dialysis, uremia, HIV, malignancy, and gastroplasty with postoperative vomiting^[15,18,19].

WE is a life-threatening condition that is avoidable by early recognition and administration of thiamine. Recognition of this disorder remains difficult because very few patients actually present with the classic signs of nystagmus, ataxia, and global confusion. To deal with this diagnostic

problem for chronic alcoholics, new operational criteria were published in 1997. The diagnosis criteria require 2 of the following 4 signs: dietary deficiencies, oculomotor abnormalities, cerebellar dysfunction, and either an altered mental state or mild memory impairment^[20]. Harper *et al*^[3] indicated that only about 16% had this classic triad, and 19% had no clinical signs. On Harper's review of both clinical and pathology studies, a wide range of the presentation with these signs was revealed. Ocular signs were present in 29% to 93%, ataxia in 23% to 70%, and mental changes in 82% to 90%.

In fact, none of the CT studies helps clinicians with the diagnosis. MRI can reveal areas of signal change in the paraventricular regions of the thalamus and in the paraventricular regions of the midbrain with this disorder. Dilation of the third ventricle and atrophy of the mamillary bodies can also be seen. MRI is helpful in confirming the diagnosis of WE; however, the absence of abnormalities does not exclude the diagnosis. The sensitivity of MRI is 53%, whereas the specificity is 93%^[21-24].

WE is a medical emergency and treatment consists of hospital admission and administration of intravenous thiamine. The clinical response after administering thiamine is usually striking and rapid enough to be virtually diagnostic^[3]. As few as 2 mg of thiamine may be enough to reverse the ocular symptoms (which generally begin to improve in 1-6 h), however, initial doses of at least 100 mg are usually administered. Ataxia and acute confusional state may resolve dramatically, although improvement may not be noted for days or months. We suggest starting thiamine prior to treatment with IV glucose solutions, and continuing until the patient resumes a normal diet. Magnesium is an indispensable cofactor in thiamine-dependent metabolism. In hypomagnesemic states, normal function of thiamine pyrophosphate, the active coenzyme containing thiamine, does not occur. Consequently, the final step in treating WE is correcting magnesium deficiency. The prognosis of WE depends on the stage of disease and prompt institution of thiamine^[15,16].

Our study revealed that WE continued to be a rare but life-threatening condition often overlooked in the course of AP. WE occurred in restoration stage of SAP/AP. Ocular abnormalities were the hallmarks of WE, and horizontal nystagmus was common. Long fasting, hyperemesis and TPN without Vit B1 in the course of AP were main causes of Vit B1 deficiency. Two patients with WE were treated with parenteral Vit B1 and survived; two patients once misdiagnosed as PE or cephalitis.

Chen *et al*^[1] analyzed 185 patients with AP complicated with encephalopathy. They thought that encephalopathy appearing in the early course of AP was PE. PE had a high mortality of 57%. WE appeared in the late course of AP (> 2 wk or in recovery period), and had a mortality of 33% (26/78). The difference between the two groups was significant ($P < 0.01$). Supplement of thiamine in time resulted in lower mortality in WE. Therefore, they suggested that patients who have been on fasting for a long time (more than 10 d) should be given thiamine intramuscularly in case WE occurs.

Winslet *et al*^[25] reported a young obese female with AP complicated by pseudocyst formation and intermittent

gastric outlet obstruction, who had been maintained on high-calorie enteral feeds, developed a sudden onset of confusion and ophthalmoplegia associated with papilloedema and retinal haemorrhages. A possible diagnosis of WE was made. The patient was treated with parenteral thiamine, and survived. The authors suggested that any patient with suspicious or unusual neurological symptoms and signs associated with possible malnutrition, hyperemesis or malabsorption should be given intravenous thiamine without delay to avoid the potential morbidity and mortality associated with undiagnosed WE.

In summary, it is difficult to diagnose earlier PE and WE complicating AP. In case differential diagnosis of PE and WE is baffled, Vit B1 diagnostic treatment may be useful: patients' condition of WE is supposed to improve after injected Vit B1 (100 mg/d) therapy for 1-3 d. If a patient, in the course of pancreatitis, has suspicious or unusual neurological symptoms and signs, a possible diagnosis of encephalopathy should be made, and the patient should be given intravenous thiamine without delay to avoid the potential morbidity and mortality associated with undiagnosed WE.

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RAPID COMMUNICATION

Disordered beta-catenin expression and E-cadherin/CDH1 promoter methylation in gastric carcinoma

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variant gastric carcinoma. Methylation of CDH1 gene in the absence of E-cadherin is an early event in gastric carcinogenesis.

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Key words: Gastric carcinoma; Beta-catenin; E-cadherin; DNA methylation

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Abstract

AIM: To investigate the distribution of beta-catenin in nuclei or membrane/cytoplasm of gastric carcinoma cells, the relationship between E-cadherin gene methylation and its expression, and the role of beta-catenin and E-cadherin as potential molecular markers in predicting tumor infiltration.

METHODS: Twenty-nine cases of gastric carcinoma, classified as diffuse and intestinal variants, were selected for study. Nuclear and cytoplasmic proteins were purified and beta-catenin content was detected by ELISA. DNA methylation of E-cadherin/CDH1 gene promoter was studied by methylation-specific PCR and compared with E-cadherin expression detected by immunohistochemistry.

RESULTS: In 27 cases of gastric carcinoma, the ratio of beta-catenin content between nuclei and membrane/cytoplasm was correlated with the T-classification ($r = 0.392$, $P = 0.043$). The significance was present between T2 and T3 groups. No correlation was detected between diffuse and intestinal variants in terms of their beta-catenin distribution. In 21 cases of diffuse variants of gastric carcinoma, there was a difference in E-cadherin expression between CDH1 gene-methylated group and non-methylated group (29 % vs 71 %, $P = 0.027$). No correlation between CDH1 gene methylation and T-classification was found, neither was the significance between E-cadherin expression and tumor infiltration grade.

CONCLUSION: Comparative analysis of nuclear and membrane/cytoplasmic beta-catenin can predict local tumor infiltration. E-cadherin/CDH1 gene methylation is an important cause for its gene silence in diffuse

INTRODUCTION

Gastric carcinoma is highly malignant and usually results in a poor outcome. Until now there is no satisfactory tumor marker for predicting its evolution. E-cadherin, a trans-membrane glycoprotein of 120 KD which is expressed in normal epithelium, may play an important role both in cell-cell adhesion and in tumor invasion and metastasis. The low expression of E-cadherin may favor the dissociation of carcinoma cells from one another for their invading out of basal membrane. Though mutation and allelic loss have been confirmed as major mechanisms for E-cadherin (CDH1) gene inactivation in many malignancies^[1], it has been recently reported that CDH1 promoter methylation could be frequently detected in gastric carcinoma^[2]. Still it is controversial whether DNA methylation is the main cause for E-cadherin/CDH1 gene silence. As a copartner of E-cadherin, beta-catenin is critical for intercellular adhesion in membrane and cytoplasm of cells, it also plays a role as a transcription activating protein in nuclei^[3]. The nuclear accumulation of beta-catenin may stimulate gastric epithelium proliferation^[4], nevertheless the effect of beta-catenin on tumor infiltration in gastric carcinoma is waiting to be more precisely studied by quantitative analysis. The aim of the present study was to investigate the relationship between E-cadherin gene methylation and its expression, the distribution of beta-catenin in nuclei and cytoplasm in gastric carcinoma, and the role of beta-catenin and E-cadherin as potential molecular markers in predicting tumor infiltration.

MATERIALS AND METHODS

Patients and tumor samples

Tissue blocks were obtained from the Department of Digestive Disease, Shanghai Ruijing Hospital and the Department of Pathology, Shanghai No.2 Hospital, involving 29 cases of gastric carcinoma operated from 2002 to 2003, of which 21 were diffuse variants and 8 were intestinal variants, together with 5 paraneoplastic non-tumor gastric tissues. The samples were freshly frozen at -70°C for DNA and protein extraction. Also the samples were fixed in 40 g/L formalin buffer then paraffin-embedded routinely for immunohistochemistry. T-classification revealed that 3 cases were T1, 5 T2, 18 T3 and 3 T4.

ELISA for nuclear/cytoplasmic beta-catenin

Nuclear and cytoplasmic protein was isolated for beta-catenin analysis. Frozen tumor tissue (1.0 cm × 1.0 cm × 1.0 cm) from each case was cut into minimal sections and homogenized manually for 5 min at 4°C with 700 µL cytoplasmic lysis buffer (0.15 mol/L NaCl, 10 mmol/L HEPES, 1mmol/L EDTA, 6 mL/L NP-40). Membrane and cytoplasmic lysis were checked by microscopic examination. The nuclei were collected by centrifuging for 5 min at 1 300 r/min at 4°C, then vigorously homogenized for 30 min at 4°C with 500 µL nuclear lysis buffer (0.4 mol/L NaCl, 20 mmol/L HEPES, 0.2 mmol/L EDTA, 0.5 mmol/L PMSF, 250 mL/L glycerol, 1.2 mmol/L MgCl₂, 0.5 mmol/L DTT, 0.5 mg/L leupeptin, 0.5 mg/L aprotinin, 0.5 mg/L pepstatin). The protein in nuclear or cytoplasmic solution was tested by Coomassie reagents following the manufacturer's instructions (Coomassie Plus-200 protein assay reagent, No-23238, Hyclone-PIERCE, USA).

Nuclear or cytoplasmic content of beta-catenin was analyzed by ELISA. The proteins were immobilized onto 96-well microtiter plates at 4°C, and washed with PBS-0.5 mL/L Tween 20. Monoclonal mouse antibody (anti-beta-catenin, M-0545, Antibody Diagnostica Company, USA) was applied in 1:25 dilution of PBS-0.5 mL/L Tween 20 at 37°C for 1h. After being washed, the wells were incubated with AKP-conjugated secondary antibody and then washed again and 1mg/mL pNPP was added. Absorbance of eluted dye was measured at A312nm. Negative controls were performed by replacing primary antibody with PBS-0.5 mL/L Tween 20.

Methylation-specific PCR (MSP) for E-cadherin/CDH1 gene

For each sample, the frozen gastric tissue (0.5 cm × 0.5 cm × 0.5 cm) was cut into minimal sections and incubated with proteinase K (20 g/L) at 55°C for 24 h. The DNA was extracted by standard phenol/chloroform technique.

DNA samples (100 µL) were incubated in 0.2 mol/L NaOH at 37°C for 10 min, then modified with sodium bisulfite solution following the manufacturer's instructions (CpGenome DNA modification kit, S7820, Ingergen Company). The modified DNA was amplified with E-cadherin/CDH1 gene-specific primers as follow: methylated-specific primer set: sense 5'-GGTGAATTTTGTAGTTAATTAGCCGGTAC-3' and antisense 5'-CATAACTAACCG AAAACGCCG-3', yielding a

product of 204 bp; unmethylated-specific primer set: sense 5'-GGTAGGTGAATTTTGTAGTTAATTAGTGGTA-3' and antisense 5'-ACCCATAACTAACCAAAAACACCA-3', yielding a product of 211 bp^[5]. The PCR mixture (50 µL in total) contained 1 × buffer (SABC Biochemical) with 1.5 mmol/L MgCl₂, 0.2 mmol dNTPs, 0.2 µmol of each primer, and 4 µL of DNA sample. PCR conditions were 10 min at 94°C, after which 3U of Taq DNA polymerase (SABC Biochemical) was added, and 35 cycles at 94°C for 50 s, at 57°C for 40 s, at 72°C for 90 s, and a final extension at 72°C for 5 min. The positive control was performed on DNA from normal gastric tissue by using unmethylated-specific primers, and the negative control was prepared on PCR mixture without primers. The PCR products were migrated by electrophoresis on 20 g/L agarose gel, with 100 bp DNA ladder as a DNA marker.

Immunohistochemistry (IHC) for E-cadherin expression

Formalin-fixed paraffin-embedded tissue sections (4 µm thick) were deparaffinized in xylene for 10 min, and rehydrated through graded alcohols to water. Antigen retrieval was performed by microwave of tissue sections in 10 mmol/L sodium citrate buffer (pH 6.0) for 15 min at 750 W. Endogenous peroxidase activity was blocked with 3 mL/L hydrogen peroxide. Primary antibody (anti-E-cadherin, mouse monoclonal antibody, Antibody Diagnostica Company, USA) was applied at 1:25 dilution and incubated for 1 h at room temperature. The slides were washed in PBS for 15 min, and secondary incubations were carried out by using anti-mouse antibody-polymerized dextran-HRP complex (ADI Two-Step System, Antibody Diagnostica Company, USA) for 30 min. Immunoreactivity was visualized with 3,3'-diaminobenzidine tetrahydrochloride (DAB), and counterstained with hematoxylin. The brown-stained color in cell membrane by DAB was defined as positive reactivity. Negative controls were performed by replacing primary antibody with PBS. The DAB staining in nuclei or cytoplasm was considered as negative.

The expression of E-cadherin was considered as positive when at least 10% of tumor cells were colored by DAB^[6]. In quantitative evaluation, 5 microscopic fields were analyzed on each tissue slide. The percentage of positive cells (300 to 900 tumor cells counted for each sample) was classified as grade 1 (11%-25%), grade 2 (26%-50%), grade 3 (51%-75%) and grade 4 (more than 75%).

Statistical analysis

Fisher's exact probability test was used to evaluate the relation between E-cadherin/CDH1 gene methylation and its expression. Correlations among the E-cadherin protein, the ratio of beta-catenin content between nuclei and membrane/cytoplasm, the tumor infiltration grading and the Lauren's typing were evaluated by method of Spearman (statistical software: SAS 6.12).

RESULTS

ELISA for nuclear/cytoplasmic beta-catenin

Twenty-seven cases of gastric carcinoma were successfully analyzed for beta-catenin distribution. The ratio of beta-catenin content between nuclei and membrane/cytoplasm

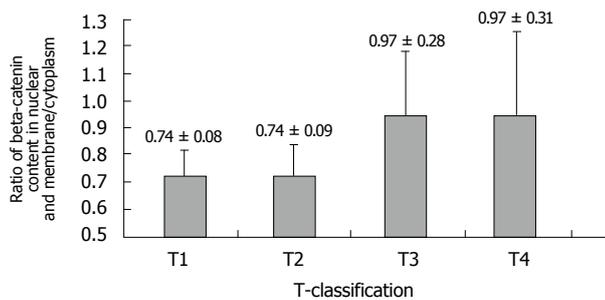


Figure 1 Ratio of beta-catenin content between nuclei and membrane/cytoplasm in different groups of gastric carcinoma according to the T-classification levels.

was correlated with the T-classification ($r = 0.392$, $P = 0.043$). The significance was present between T2 and T3 groups (Figure 1). There was no relation between diffuse variant and intestinal variant in terms of their beta-catenin distribution in tumor cells.

Methylation-specific PCR for E-cadherin/CDH1 gene

In 29 cases of gastric carcinoma, CDH1 gene promoter methylation was identified in 13 cases (45%) (Figure 2), of which 7 cases were histologically diffuse variants (33%), 6 cases intestinal variants (75%). All 3 T1 cases were found to be positive for CDH1 gene methylation, so did 1 of 5 T2 cases, 7 of 18 T3 cases and 2 of 3 T4 cases. There was no difference in diffuse and intestinal variants for their CDH1 gene methylation, and no correlation between CDH1 gene methylation and T-classification was found. The CDH1 gene methylation was also identified in 3 specimens of paraneoplastic non-tumor gastric tissues.

Immunohistochemistry for E-cadherin expression

E-cadherin expression was detected in 18 of 29 gastric carcinoma cases (62%) (Figure 3), of them 12 cases were diffuse variants (57%) and 6 cases intestinal variants (75%). Two of 3 T1 cases, 2 of 5 T2 cases, 11 of 18 T3 cases and all 3 T4 cases were found to be positive for E-cadherin expression.

Of the 13 gastric carcinoma cases which were identified for CDH1 gene methylation, E-cadherin expression was found in 2 of 7 diffuse variants and 5 of 6 intestinal variants. In 21 cases of diffuse variants, there was a difference in E-cadherin expression between CDH1 gene-methylated group and non-methylated group (29% vs 71%, $P = 0.027$). No significance between E-cadherin expression and T-classification was found, neither was the relation between diffuse variant and intestinal variant for their E-cadherin expression.

DISCUSSION

Our previous study demonstrated that E-cadherin and beta-catenin are co-expressed in gastric carcinoma^[7]. The regulation at their transcriptional levels seems to be independently controlled by different mechanisms^[8]. Beta-catenin is a bifunctional protein, location in nuclear or cytoplasm/membrane is critical for its activity as an adhesive factor or proto-oncoprotein. In nuclei, beta-catenin plays a role as a target of the wnt signaling pathway in stimulat-

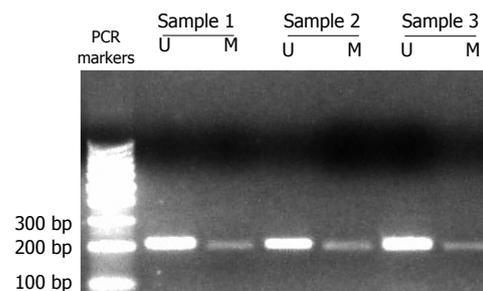


Figure 2 Methylation-specific PCR (MSP) analysis of E-cadherin/CDH1 gene in gastric carcinoma. Sodium bisulfite-modified DNA samples were subjected to PCR with specific primers. Existence of methylation was demonstrated by the presence of PCR product (204 bp) rendered by methylated specific primers. M: methylated-specific primer sets; U: unmethylated-specific primer sets.

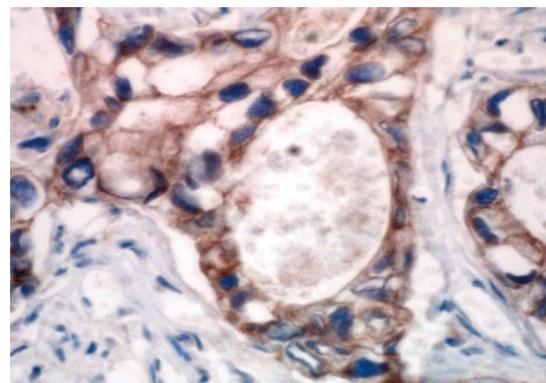


Figure 3 Immunohistochemical analysis of E-cadherin expression in gastric carcinoma (DAB × 400). Positive reaction was assessed as membrane reactivity.

ing cell proliferation through activation of cyclin D1^[9] and c-myc gene^[10]. The nuclear translocation of beta-catenin depends on its stabilization and the P13K signaling pathway. The latter may be activated by EB virus infection^[11], which is also thought to be an important cause for E-cadherin/CDH1 gene methylation^[12].

The nuclear accumulation of beta-catenin seems to be helpful, regarding tumor infiltration in gastric^[13] and colorectal carcinoma^[14], but this accumulation could also be found in early neoplasia or even in non-tumor metaplasia^[4], and beta-catenin expression at cell level does not appear to change from normal to carcinoma epithelium^[15]. Our results indicated that the evaluation of both nuclear and membrane/cytoplasmic beta-catenin expression might have a prognostic implication and a potential diagnostic value for gastric carcinoma. In the present study, we found a significant variation of beta-catenin distribution from T2 to T3 grade, suggesting that beta-catenin plays an important role in tumor infiltration in gastric smooth muscle. The possible mechanism may be that beta-catenin could regulate the matrix metalloproteinase activity in degradation of extracellular matrix components^[16], and this regulation is mainly focused on local infiltration rather than metastasis^[17].

E-cadherin is an indispensable protein for cell adhesion in normal epithelium. Loss of its function is propitious to tumor invasion. For silence of the E-cadherin/CDH1 gene in gastric carcinoma, recent studies showed that not only the changes of DNA sequence but also the epigenetic

modification^[18,19] such as DNA methylation, may play an important role in the loss of E-cadherin expression.

DNA methylation has attracted great attention recently, as many tumor suppresser genes contain CpG-rich promoters called CpG islands, which are normally unmethylated. Silence of these genes may occur in association with the aberrant hypermethylation of their promoter regions. It has been reported that E-cadherin/CDH1 gene is frequently methylated in colorectal and gastric carcinoma, but its endogenetic changes such as mutation are rare^[12,20]. An *in vitro* study demonstrated that the E-cadherin expression, associated with the cell metamorphosis, could be induced by demethylation drugs^[21]. These studies indicate that DNA methylation may be a possible mechanism for E-cadherin/CDH1 gene silence in human gastric carcinoma.

In the present study, 45% of gastric carcinomas were positive for E-cadherin/CDH1 gene methylation, which corresponds to the result of previous study^[22]. We showed that E-cadherin expression was negatively correlated with its gene methylation in diffuse variants, indicating that DNA methylation may diminish E-cadherin expression and thus favor the devastating activity of the tumor. The data reported here do not provide evidence of correlation between CDH1 gene methylation and tumor infiltration, but are in agreement with the hypothesis that methylation status of CDH1 promoter may be an early event in gastric carcinogenesis^[15].

In conclusion, comparative analysis of nuclear and membrane/cytoplasmic beta-catenin can predict tumor infiltration in gastric wall, and the E-cadherin/CDH1 gene methylation is an important cause for its gene silence in diffuse variant gastric carcinomas. Analysis for detecting distribution of beta-catenin protein and methylation state of E-cadherin/CDH1 gene helps to understand the dynamic process of oncogenesis.

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RAPID COMMUNICATION

Effect of Tetrandrine on LPS-induced NF- κ B activation in isolated pancreatic acinar cells of rat

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Abstract

AIM: To investigate the effect of Tetrandrine (Tet) on LPS-induced NF- κ B activation and cell injury in pancreatic acinar cells and to explore the mechanism of Tetrandrine preventing LPS-induced acinar cell injury.

METHODS: Male rat pancreatic acinar cells were isolated by collagenase digestion, then exposed to LPS (10 mg/L), Tet (50 μ mol/L, 100 μ mol/L) or normal media. At different time point (30 min, 1 h, 4 h, 10 h) after treatment with the agents, cell viability was determined by MTT, the product and nuclear translocation of subunit p65 of NF- κ B was visualized by immunofluorescence staining and nuclear protein was extracted to perform EMSA which was used to assay the NF- κ B binding activity.

RESULTS: LPS induced cell damage directly in a time dependent manner and Tet attenuated LPS-induced cell damage (50 μ mol/L, $P < 0.05$; 100 μ mol/L, $P < 0.01$). NF- κ B p65 immunofluorescence staining in cytoplasm increased and began showing its nuclear translocation within 30 min and the peak was shown at 1 h of LPS 10 mg/L treatment. NF- κ B DNA binding activity showed the same alteration pattern as p65 immunofluorescence staining. In Tet group, the immunofluorescence staining in cytoplasm and nuclear translocation of NF- κ B were inhibited significantly.

CONCLUSION: NF- κ B activation is an important early event that may contribute to inflammatory responses and cell injury in pancreatic acinar cells. Tet possesses the protective effect on LPS-induced acinar cell injury by inhibiting NF- κ B activation.

INTRODUCTION

Many animal and clinical studies have shown that once the progress of acute pancreatitis is initiated, common inflammatory responses are involved. There is a local inflammatory reaction at the site of injury; if marked, this leads to a systematic inflammatory response syndrome (SIRS), and this systemic response is ultimately responsible for the majority of the mortality^[1,2]. Lipopolysaccharides (LPS) have been found in the plasma of patients suffering from severe pancreatitis at an early stage of the disease and inflammatory changes resembling acute pancreatitis were described after administration of LPS to several animal species^[3,4].

The transcription factor nuclear factor- κ B (NF- κ B) is a key regulator of cytokine induction. NF- κ B represents a family of proteins sharing the Rel homology domain, which bind to DNA as homo- or heterodimers, and activate a multitude of cellular early and stress-related response genes, such as the genes for cytokines, adhesion molecules^[5]. NF- κ B exists in an inactive form in the cytoplasm of most cells where it binds to an inhibiting protein, I κ B. Many stimuli activate NF- κ B, including cytokines, LPS and oxidative stress. Stimuli trigger the translocation of NF- κ B from cytosol to nucleus where NF- κ B binds to its consensus sequence on the promoter-enhancer region of different genes and regulates transcription of specific genes^[6]. Recently, some experimental results suggested that the cholecystokinin (CCK) analogue cerulein induced the rapid activation of NF- κ B both *in vivo* and in cultured acinar cells *in vitro* and activation of NF- κ B preceded pancreatic injury and inflammation^[7]. Early blockage of NF- κ B activation improved the survival of rats with taurocholate pancreatitis^[8].

In our previous study^[9,10], we have found that LPS could directly induce the calcium overload, NF- κ B activation and cell injury in isolated rat pancreatic acinar cell. Furthermore, NF- κ B activation could be attenuated by EGTA, a calcium chelater. Based on these results, we speculate that calcium disorder might take part in the NF- κ B activation in isolated rat pancreatic acinar cell and calcium channel blocker (CCB) may be useful for the treatment of acute pancreatitis by influencing NF- κ B activation in pancreatic acinar cells.

Tetrandrine (Tet), is a main component of fourstamen stephania root which belongs to traditional Chinese drug and one of natural non-specific CCBs. Tet possesses complicated pharmacological effects and has been used in the therapy of many kinds of disease, such as hypertension, arrhythmia, hepatic fibrosis, *etc*^[11]. In former *in vivo* experiments^[12,13], we have observed that Tet could improve the pathological alteration of pancreas and lung and decrease the mortality of rats with acute taurocholate pancreatitis. In an attempt to further explore the mechanism of Tet in the treatment of AP, we adopted isolated pancreatic acinar cells and examined whether Tet possesses the protective effect on LPS-induced acinar cell injury by inhibiting NF- κ B activation.

MATERIALS AND METHODS

Animals and materials

Male Sprague-Dawley rats (200–250 g) obtained from Experimental Animal Center of Chinese Academy of Sciences (Grade SPF II Certificate No. SYXK 2002-0023) were fasted for 12 h. *Escherichia coli* LPS (WE coli 055: B5) and MTT were purchased from Sigma Co, USA. Tetrandrine was provided by Pharmacology Department of 2nd Medical University of PLA. NF- κ B gel shift assay system kits were provided by Promega Co, USA. [γ -³²P] ATP was purchased from Shanghai Isotope Company. Antibody against NF- κ B p65 was obtained from Santa Cruz Biotechnology Co, CA. Fluorescein isothiocyanate (FITC)-conjugated goat-anti-rabbit antibody was purchased from KPL Company, USA. All other chemicals were supplied from local source at the highest purity available. HEPES buffer salt solution (HBSS) (in mmol/L): NaCl 118, KCl 4.7, CaCl₂ 2.5, MgCl₂ 1.13, NaH₂PO₄ · 2H₂O 1.0, D-glucose 5.5, HEPES 10, bovine serum albumin 2 g/L, minimum essence medium 2%, L-glutamine 2.0, soybean trypsin inhibitor 0.1 g/L, pH adjusted to 7.4 with NaOH 4 mmol/L.

Preparation of isolated pancreatic acinar cells

Pancreatic acinar cells were isolated from male SD rats by collagenase digestion^[14]. In brief, after the rat was anesthetized, the pancreas was quickly removed and parenchyma was minced into small fragments and incubated in 10-mL standard buffer containing collagenase V (90 kU/L) at 37°C, and the pancreatic fragments were digested again by collagenase under a shaking condition for 20 min in an incubator. After collagenase digestion, tissue was gently pipetted. Dispersed acini were filtered through a 150- μ m nylon mesh, centrifuged 3 times each for 3 min at 100 \times g, resuspended with culture media (in HBSS, replacing bovine serum albumin 2 g/L with 10%

heat-activated bovine serum) and incubated with 95% O₂, and 5% CO₂.

Cell culture and treatment

Pancreatic acinar cells were planted in 24 and 96 well plates at 37°C in a CO₂ (50 mL/L) incubator and cultured for 4 h, then exposed to different content of media (10 mg/L LPS, 50 μ mol/L Tet and 100 μ mol/L Tet or culture media as control) for 30 min, 1 h, 4 h and 10 h respectively.

MTT assay

An MTT assay was employed to assess the viable cell number quantitatively. Briefly, 100 μ L of cell suspension (1×10^4 cells) was seeded into 96-well tissue-culture plates. Cells were treated with LPS (10 mg/L), or normal media. Cells in Tet group were treated with 50 μ mol/L or 100 μ mol/L Tet for 15 min before stimulated by LPS. After treatment with these agents for indicated period, 10 μ L MTT (terminal concentration 0.5 g/L) was added into each well, and incubated for 4 h. The formazan crystals were produced by viable cells and dissolved by Me₂SO, and the optical density (OD) of the solution was measured at 490 nm of wavelength. Cell viability was directly proportional to OD value. The viable cell number was expressed as a percentage relative to control cells, measured as $100\% \times OD_{490, \text{treated}} / OD_{490, \text{control}}$ (at 0 h timepoint).

NF- κ B immunofluorescence studies

The pancreatic acinar cells that were seeded onto 24-well plates and treated as described above respectively for indicated period were used for immunofluorescence staining. First, the glass slips were pretreated with 0.1% poly-lysine and cell suspensions were incubated at room temperature for 60 min. The cells were then fixed in freshly prepared 4% phosphate-buffered paraformaldehyde for 20 min at 4°C. Then they underwent permeabilization with the addition of phosphate-buffered 0.1% Triton X-100 (wt/vol) for 5 min at room temperature and then incubated in PBS containing 1% bovine serum albumin (wt/vol, blocking solution) for 30 min, also at room temperature. Incubation of the cells was done with either rabbit anti-rat NF- κ B p65 polyclonal antibodies (1:100 dilution in blocking solution) or with blocking solution alone as negative control for 1 h at room temperature. This was followed by incubation with goat anti-rabbit antibodies (1:100 dilution in blocking solution) conjugated to FITC for 45 min at room temperature. Finally, the slips were mounted and sealed for examination under a confocal microscope.

Electrophoretic mobility shift assay (EMSA) for determination of NF- κ B activity

After treatment with stimuli for the above indicated period, pancreatic acinar cells were collected to extract nuclear protein for further examination of the activity of NF- κ B by EMSA.

Nuclear protein extraction: Nuclear protein extracts were prepared according to Steinle *et al*^[6] with the following modifications. Pancreatic acinar cells (4×10^6) were collected, washed twice with cold phosphate-buffered sa-

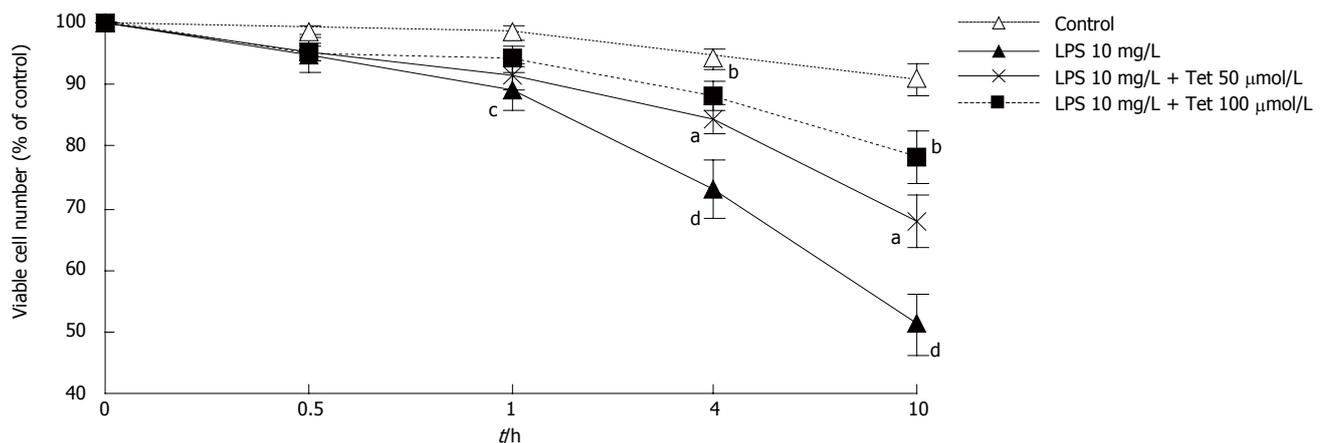


Figure 1 Protective effect of Tet on LPS-induced cell damage in pancreatic acinar cells. Pancreatic acinar cells were treated with LPS 10 mg/L, Tet 50 μ mol/L or Tet 100 μ mol/L for 1, 4, and 10 h. Results are expressed as percentage of control values (without LPS treatment) at 0 h time-point ($n = 3$). Mean \pm SD. ^a $P < 0.05$, ^b $P < 0.01$ vs LPS group at the same time-point. ^c $P < 0.05$, ^d $P < 0.01$ vs control group at the same time-point.

line (PBS), homogenized in 1.5% citrate, then centrifuged at 4000 g for 15 min at 4°C, at which point the supernatant was removed. Then 0.3 mL of 0.25 mol/L sucrose-citrate was added into the pellet and mixed to create a nucleic suspension, which was pipetted and paved on the surface of 1.2 mL 0.88 mol/L sucrose-citrate in a tube and centrifuged at 2000 g for 10 min. The resulting pellet was washed twice in 0.05 mol/L Tris-HCl (pH 7.5)-0.15 mol/L NaCl and centrifuged at 2000 g for 10 min at 4°C. After removing the supernatant, an equal volume of KMTD (0.3 mmol/L KCl, 1 mmol/L MgCl₂, 10 mmol/L Tris-HCl (pH 8.0) and 1 mmol/L DTT) was mixed into the pellet and incubated for 1 h. During the incubation, the samples were shaken drastically every 15 min. After incubation, the suspension was centrifuged at 15000 g for 15 min at 4°C. Aliquots of the nuclear protein extracts from supernatant were stored at -70°C. Protein content of the extracts was determined using Lowry method with bovine serum albumin as the standard.

EMSA: Activity of NF- κ B was examined by EMSA^[15]. The NF- κ B oligonucleotide contains DNA binding sites for NF- κ B transcription factors. The double-stranded DNA probe sequence is 5'AGT TGA GGG GAC TTT CCC AGG C 3' and antisense 3'TCA ACT CCC CTG AAA GGG TCC G 5' (the binding site is underlined). The 3.5 pmol of the appropriate consensus oligonucleotide was end-labeled with [γ -³²P] ATP using T₄ polynucleotide kinase. The ³²P-labeled double stranded oligonucleotide was used as a specific probe. For the competition assay, the unrelated oligonucleotide, AP₂ consensus oligonucleotide which lacked κ B binding site, was used as a non-specific probe. Nuclear proteins extracted from HeLa cell were used as positive control. The nuclear extract equivalent to 5 μ g protein was incubated with radiolabeled probe in reaction buffer, and then the mixture was subjected to electrophoresis on 7% acrylamide (wt/vol) gel at 250 V in 0.5 \times TBE buffer for 2 h. After being dried, the gel was exposed to X ray film at -70°C for 48 h.

Statistical analysis

Each n refers to the number of separated experiment. The

quantitative data were expressed as mean \pm SD and compared using unpaired t -test. The significant differences of incidence of nuclear translocation of NF- κ B p65 between LPS group and Tet-pretreated group were analyzed by Chi-square test. $P < 0.05$ was considered significant.

RESULTS

Tet attenuated LPS-induced cell damage

The viable cell number exposed to LPS 10 mg/L decreased with the increase of stimulating time and the difference was significant ($P < 0.05$), compared with control at the same time point. Compared with LPS group, cell mortality in the group pretreated with Tet at 50 μ mol/L or 100 μ mol/L decreased significantly at each time point (50 μ mol/L, $P < 0.05$, 100 μ mol/L, $P < 0.01$, Figure 1). It suggests that LPS induced cell damage in a time-dependent manner and Tet could attenuate LPS-induced cell damage.

Tet abrogated LPS-induced NF- κ B product and nuclear translocation in pancreatic acinar cells

To monitor its activity at a cellular level, NF- κ B p65 antibody was used to recognize the product and nuclear translocation signal of the p65. Active p65 level in cytoplasm and its nuclear translocation in pancreatic acinar cells were monitored by immunofluorescence visualization. The cells untreated with LPS showed a slight exclusive cytoplasmic fluorescence pattern (Figure 2B). In contrast, cytoplasmic fluorescence in pancreatic acinar cells treated with LPS 10 mg/L for 1 h and 4 h increased significantly (Figure 2C and 2D). P65 nuclear translocation was seen in > 60% of pancreatic acinar cells incubated with LPS for 30 min and nuclear staining further increased and reached the peak after 1 h of treatment in the LPS group (nuclear translocation ratio > 90%). In the Tet-pretreated group, the pancreatic acinar cells were pretreated with Tet for 15 min and then stimulated with 10 mg/L LPS for 30 min, 1 h and 4 h. Cytoplasmic fluorescence in pancreatic acinar cells was impaired (Figure 2E and 2F) and P65 nuclear translocation was little seen, compared with that in the LPS group ($P < 0.05$ or $P < 0.01$, Table 1).

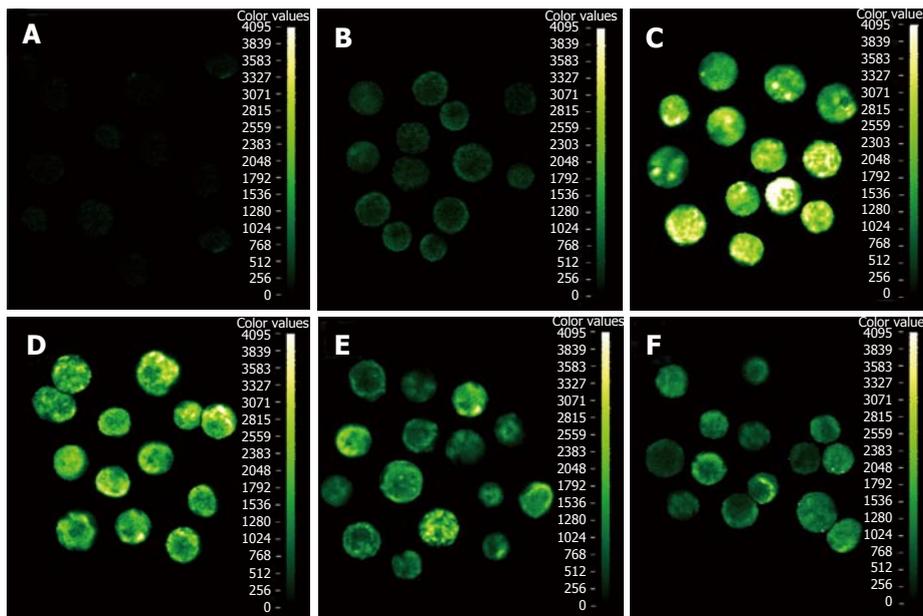


Figure 2 Tet prevented LPS-induced nuclear translocation of p65 in isolated pancreatic acinar cells. P65 was visualized by indirect immunofluorescence staining using rabbit anti-p65 polyclonal antibodies (1:100) which only recognized NF-κB p65. Goat anti-rabbit antibodies (1:100) conjugated to FITC was performed, and visualized under a confocal microscope. Nuclear p65 was observed at 1 h (C), 4 h (D) after treatment with LPS (10 mg/L) but not in saline-treated controls at 1 h (B), nor in negative control (A). Nuclear localization of p65 and the fluorescence in cytoplasm were markedly reduced by Tet 100 μmol/L at 1 (E), 4 h (F), compared with that of LPS group at the same time (Original magnification × 400).

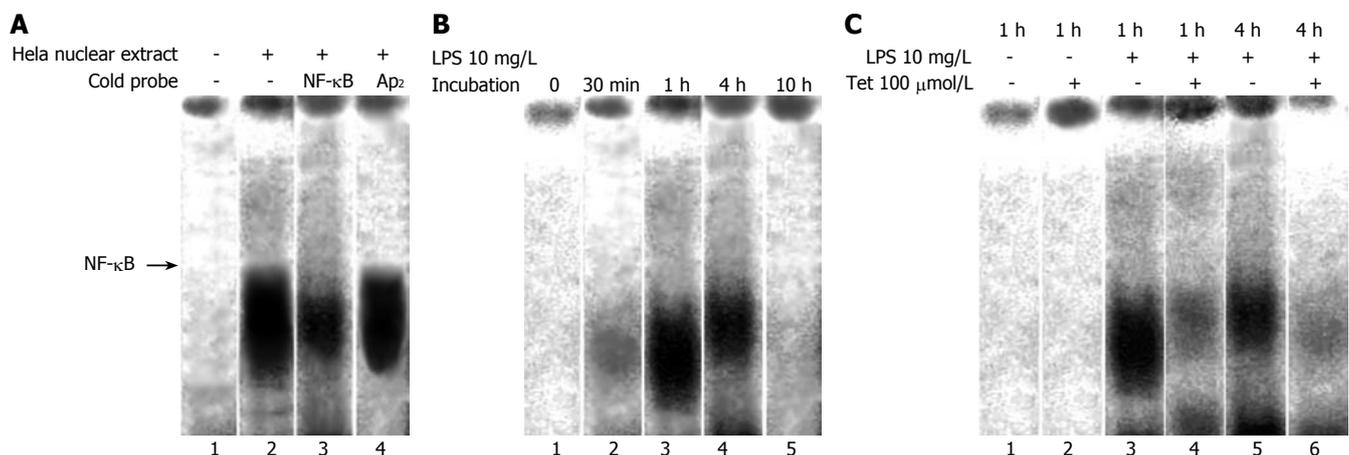


Figure 3 **A:** Effect of LPS on NF-κB activity in pancreatic acinar cell. Lane 1: control; Lane 2: treated with LPS 10 mg/L for 1 h; Lane 3: treated with LPS 10 mg/L for 1 h + cold NF-κB probe; Lane 4: treated with LPS 10 mg/L for 1 h + labeled AP₂ probe; **B:** The time course of LPS-induced NF-κB activity in pancreatic acinar cell. NF-κB activity in pancreatic acinar cell stimulated by LPS 10 mg/L for indicated periods of time. Lane 1: 0 min; Lane 2: 30 min; Lane 3: 1 h; Lane 4: 4 h; Lane 5: 10 h; **C:** Effect of Tet on LPS-induced NF-κB activity in pancreatic acinar cell. Lane 1: control; Lane 2: treated with Tet 100 μmol/L for 1 h; Lane 3: treated with LPS 10 mg/L for 1 h; Lane 4: treated with LPS 10 mg/L + Tet 100 μmol/L for 1 h; Lane 5: treated with LPS 10 mg/L for 4 h; Lane 6: treated with LPS 10 mg/L + Tet 100 μmol/L for 4 h.

Tet interfered with LPS-induced NF-κB activation:

EMSA was performed to detect the binding activity of NF-κB in different group. Pancreatic acinar cells were incubated with LPS or Tet and nuclear extracts were prepared and incubated with a ³²P-labeled DNA oligonucleotide containing the recognition site of NF-κB. The specificity of the shift bands was verified by competition assays: The shift bands were suppressed by the incubation with cold NF-κB probe and not influenced by non-specific competition with labeled AP₂ probe (Figure 3A). Very little NF-κB binding activity was detected under the unstimulated condition and LPS induced NF-κB binding activity increased in a time dependent manner and NF-κB binding activity was maximal at 1 h after incubation of LPS in 10 mg/L (Figure 3B).

The effect of Tet on LPS-induced NF-κB activation was assessed in pancreatic acinar cells. It was found that in the presence of Tet 100 μmol/L, the optical density and

Table 1 Effect of Tet on LPS-induced nuclear translocation of NF-κB p65 in pancreatic acinar cells

Group	Ratio of nuclear translocation (%)		
	30 min	1 h	4 h
LPS 10 mg/L	60.9	90.5	44.7
Tet 50 μmol/L + LPS	34.7 ^a	34.5 ^b	18.9 ^b
Tet 100 μmol/L + LPS	28.6 ^b	32.1 ^b	17.3 ^b

Pancreatic acinar cells were pretreated with 50 μmol/L or 100 μmol/L Tet for 15 min and then stimulated with 10 mg/L LPS for 30 min, 1 h and 4 h. In the Tet-pretreated group, cytoplasmic fluorescence in pancreatic acinar cells was impaired (Figure 2E and 2F) and P65 nuclear translocation was little seen, compared with that in the LPS group. ^aP < 0.05, ^bP < 0.01 vs LPS group at the same time-point.

volume of NF-κB shift band markedly decreased than that of LPS group at the same time (Figure 3C).

DISCUSSION

Lipopolysaccharide (LPS, endotoxin) of the gram negative bacteria outer wall plays a central role in the pathophysiology of the sepsis syndrome^[3]. Many experiments demonstrated it is related to the pathogenesis of acute pancreatitis^[4,15,16].

LPS induces NF- κ B activation in many cells, such as canine tracheal smooth muscle cells, primary rat microglia or monocyte of humans^[17-19]. In our previous study^[9], we have found that LPS induced the increase of NF- κ B binding activity by EMSA and p65 subunit of NF- κ B translocation to nuclei by immunofluorescence. However, it is unknown whether there is a more primary factor which precedes the LPS-induced NF- κ B activation. Our other study^[10] revealed that the first alteration measured after exposing pancreatic acinar cells to LPS system was an increase in the $[Ca^{2+}]_i$ which appeared within several hundreds of second. The increase in $[Ca^{2+}]_i$ preceded all the other pathological events in the progress of LPS-induced pancreatic acinar cells damage. Intracellular calcium overload exerted an important effect on LPS-induced pancreatic acinar cells damage and egtazic acid, a Ca^{2+} chelate could attenuate the damage by inhibiting Ca^{2+} influx. The result suggested that the disorder of calcium homeostasis in pancreatic acinar cells was an important mediator of LPS-induced cell damage. Tando *et al* observed that caerulein-induced NF- κ B/Rel activation requires both Ca^{2+} and protein kinase C as messenger^[5]. We adopted calcium channel blocker (CCB) to observe its effects on the NF- κ B activation and viability of isolated rat pancreatic acinar cell.

In our present study, we found that LPS was able to induce the damage in intact pancreatic acinar cells directly at 1 h and the mortality of cell increased with the increase of concentration and stimulating time of LPS. The increase of NF- κ B activity preceded the pathological alteration in the progress of LPS-induced pancreatic acinar cells damage. Moreover, a typical CCB, Tet could abrogate LPS-induced NF- κ B nuclear translocation, interfere with the DNA binding activity of NF- κ B and attenuate cell damage of pancreatic acinar cells. The results show that NF- κ B activation is an important early event that may contribute to inflammatory response and cell injury in pancreatic acinar cells and Tet possesses the protective effect on LPS-induced acinar cell injury by inhibiting NF- κ B activation. These findings will be helpful to explain the mechanism of Tet in the treatment of AP at the biological level.

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Investigation on correlation between expression of CD58 molecule and severity of hepatitis B

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Abstract

AIM: To investigate the correlation between expression of CD58 and severity of hepatitis B.

METHODS: The level of soluble CD58 (sCD58) in serum of patients with hepatitis B was detected by enzyme-linked immunosorbent assay. The level of expression of membrane CD58 molecule in PBMC was detected by direct immunofluorescence. The levels of serumal TBIL, DBIL, IBIL, ALT and AST were detected by the automated biochemistry analyzer as well.

RESULTS: The levels of sCD58 in serum and membrane CD58 molecule in PBMC of patients with hepatitis B were significantly higher than that in normal controls ($P < 0.05$). Level of CD58 was related to the levels of serumal TBIL, DBIL, IBIL, ALT and AST.

CONCLUSION: The level of CD58 molecule (in both serum and PBMC form) of patients with hepatitis B is related to the degree of liver damage.

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Key words: Hepatitis B; CD58; Liver damage

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INTRODUCTION

Hepatitis B is caused by Hepatitis B virus (HBV), but the

pathogenesis of hepatitis B is not well understood. HBV infects human liver cells but has no direct cytopathic effect on these cells. Therefore, it is unlikely that direct viral cytotoxicity is the primary cause of pathology in vivo. Several studies have suggested that hepatitis B may be mediated in part by immunopathologic mechanisms. As one of the intercellular adhesion molecules, CD58 provided co-stimulatory signals for the activation of T lymphocyte, it plays an important role in promoting the adhesion of T cells to targeted cells^[1-3]. In this study, we used double antibody sandwich ELISA and direct immunofluorescence to analyze the levels of sCD58 in serum and the expression of CD58 on the surface of PBMC of patients with hepatitis B, and compared with those levels of healthy controls to evaluate the role of CD58 in the pathogenesis of hepatitis B.

MATERIALS AND METHODS

Sample collection and processing

Forty-three patients with hepatitis B are selected from outpatients and inpatients of the Department of Infectious Diseases of First Hospital of Xi'an Jiaotong University and Second Hospital of Xi'an Jiaotong University. The patients were divided into four groups, namely mild chronic hepatitis B group ($n = 12$), moderate chronic hepatitis B group ($n = 11$), severe chronic hepatitis B group ($n = 10$) and severe hepatitis B group ($n = 10$). Eleven healthy persons were taken as normal control group. The diagnostic code for Hepatitis B which edited by 5th Chinese Academic committee of Infection Disease and Parasite in 2000 was used as the classification criteria.

Detection of the sCD58 in serum

The serum samples were diluted 1:50 with assay buffer (5 μ L sample + 245 μ L assay buffer), and then the levels of sCD58 in the diluted samples were detected by use of double antibody sandwich ELISA following the kit instructions (Australia Science Laboratory company). The standard wells were designated by the instruction as well. The OD value of each well of the samples and the sCD58 standards was read out on an EL311 autoplater reader (BIO-TEK Instruments, American) with a test wavelength of 450 nm and a reference wavelength of 620 nm; hence the optimal concentration of sCD58 could be determined. Blank wells were used as background control.

Detection of membrane CD58 in PBMC

One ml of PBMC sample was mixed with 10 μ L of

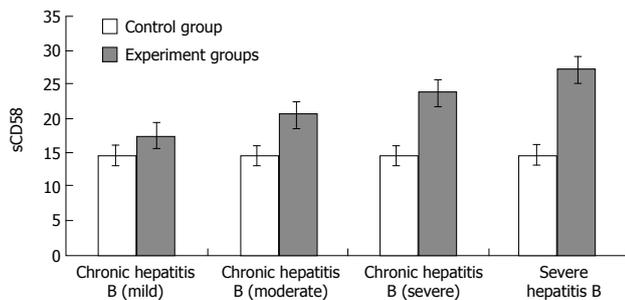


Figure 1 Content of CD58 in serum.

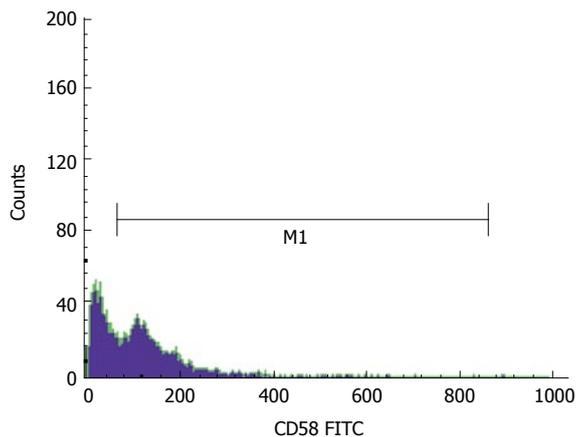


Figure 2 Content of membrane CD58 molecule in mild chronic hepatitis B.

mouse anti-human CD58 antibody marked by fluorescence FITC (American Southern Biotech), incubated in the dark at room temperature, washed twice with PBS, and centrifuged at 2500 r/min before abandoning supernatant. One ml of PBS-formaldehyde was added to fix cells, and CD58 positive cells were detected by using FACS Calibur flow cytometer.

Detection of the liver function

One mL serum sample was used to test the levels of TBIL, DBIL, IBIL, ALT and AST by the CHEMIX-180 automated biochemistry analyzer (Japanese SYSMEX Corporation).

Statistical Analysis

The significance among different groups was examined by one-way analysis of variance followed by two-sample Student's *t*-test. Differences between groups were considered significant if probability value of $P < 0.05$ were obtained. The data were presented by means \pm SD.

RESULTS

Content of sCD58 in serum

The levels of sCD58 of patient with HBV infection were significantly higher than that of normal control ($P < 0.05$); and the difference among the groups were significant ($P < 0.05$) (Figure 1).

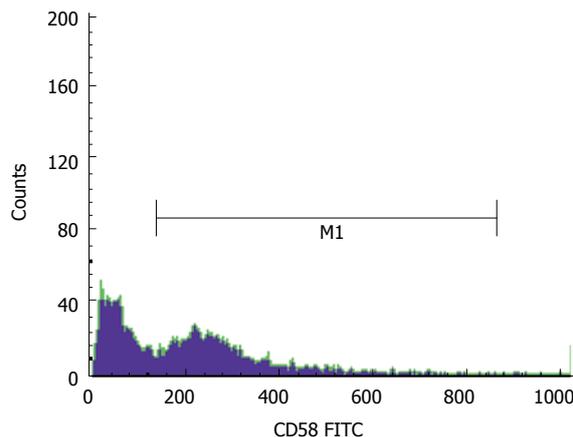


Figure 3 Content of membrane CD58 molecule in moderate chronic hepatitis B.

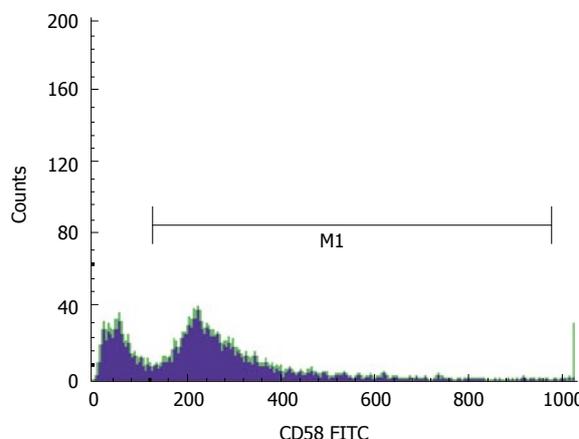


Figure 4 Content of membrane CD58 molecule in severe chronic hepatitis B.

Content of membrane CD58 molecule

The results showed that the level of membrane CD58 molecule in PBMC of patients with HBV infection was significantly higher than that of the normal group and the differences among the groups were significant ($P < 0.05$). The levels of membrane CD58 molecule increased significantly in an order from light chronic hepatitis B, medium group, severe group and severe hepatitis B. The membrane CD58 molecule in PBMC might relate to the severity of the disease (Figures 2-5, Table 1).

DISCUSSION

CD58 is also called lymphocyte function associated antigen-3 (LFA-3)^[4-6], which belongs to the CD2 family. As an important co-stimulating molecule, CD58 plays an important role in promoting the adhesion of T cells to targeted cells, and enhancing the recognition and sensitivity of T lymphocyte to the superantigen^[7-9]. CD58 promotes hyperplasia and activation of T cell^[1,2], promotes T cells soak inflammatory parts and takes part in signal transmission of T cells. Combined with CD2 molecules on the surface of NK cells, CD58 increases the adhesion between NK cells and target cells, activates NK cells^[10,11], and increases the toxin of the cells^[12,13]. After integrating

Table 1 The liver function

Group	n	TBIL ($\mu\text{mol/L}$)	DBIL ($\mu\text{mol/L}$)	IBIL ($\mu\text{mol/L}$)	ALT (IU/L)	AST (IU/L)
Normal control	11	11.25 \pm 2.14	3.12 \pm 1.54	6.41 \pm 1.85	25.19 \pm 2.58	19.57 \pm 3.06
Chronic hepatitis B (mild)	12	15.14 \pm 3.26 ^a	15.92 \pm 2.05 ^a	10.39 \pm 2.63 ^a	63.33 \pm 3.68 ^a	46.67 \pm 9.81 ^a
Chronic hepatitis B (moderate)	11	39.21 \pm 8.73 ^a	25.21 \pm 7.11 ^a	23.74 \pm 3.87 ^a	98.21 \pm 18.90 ^a	114.43 \pm 12.80 ^a
Chronic hepatitis B (severe)	10	105.33 \pm 17.67 ^a	49.88 \pm 8.62 ^a	50.86 \pm 16.05 ^a	221.61 \pm 18.19 ^a	157.01 \pm 22.54 ^a
Severe hepatitis B	10	143.57 \pm 23.15 ^a	75.26 \pm 6.56 ^a	117.35 \pm 15.27 ^a	116.73 \pm 28.57	94.82 \pm 41.49

^aP < 0.05, compared with control group.

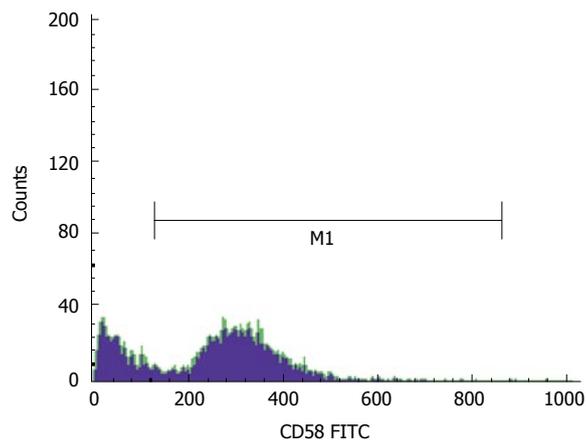


Figure 5 Content of membrane CD58 molecule in severe hepatitis B.

with activated T cells, CD58 and CD2 facilitate interferon γ and IL-2 mRNA record and translate, differentiate CD4⁺T to Th1 and further initiate the immune response of the cell^[3,14]. Some researchers proved that integrated with matching cells, CD58 may boost the ability of activated T cells and NK cells^[15,16].

Our experiment showed that the levels of sCD58 in serum and membrane CD58 molecule in PBMC of patients with HBV infection were significantly higher than that of the normal group. The levels of CD58 varied from different groups of patients with hepatitis B, correlated to the severity of the disease. The results also showed that the percentage of CD58⁺ cell of patients with hepatitis B might be related with TBIL, DBIL, IBIL, ALT and AST, which prove the expression of CD58 is closely correlated with the liver damage. According to the results of the research, the increased levels of sCD58 molecule in serum and membrane CD58 molecule in PBMC of patients with hepatitis B could enhance the adhesion of APC and T cells to identify antigen^[17,18]. This study proved that the combination of CD58 and CD2 activated T cells was similar to the way of TCR/CD3. This might lead to a series of responses in the cells, including the increase of the concentration of Ca²⁺, the activation of PKC, several lipid genes in the resting cells such as IL-2 and IFN- γ being recorded and the transformation of T cells from G0 stage to S stage. The T cells activated by integration of CD58 and CD2 would increase the record and translate of IL-2 and IFN- γ mRNA, and then differentiate into Th1 which would enhance the cell immune response. Combined

with CD2 molecules on the surface of NK cells, CD58 could activate NK cells and increase the cytotoxic effect^[19]. And this would contribute to the organisms to eliminate HBV. The cytotoxic effect of the immune cells might be implemented by releasing PF or inducing apoptosis of CD95 cells^[20].

In summary, this study showed that the sCD58 in serum and membrane CD58 molecule in PBMC of the patients with hepatitis B is related to the severity of the disease and the liver damage. CD58 might enhance the elimination of viruses through activating T and NK cells and promoting cell immune response. However, this would also lead to the damage of liver cells.

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Expression of angiopoietins, Tie2 and vascular endothelial growth factor in angiogenesis and progression of hepatocellular carcinoma

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Abstract

AIM: To investigate the significance of angiopoietins, Tie2 and vascular endothelial growth factor (VEGF) expression in the angiogenesis and progress of hepatocellular carcinoma (HCC).

METHODS: Fresh surgically resected specimens of HCC and noncancerous liver (NCL) tissue from 38 patients with HCC were obtained, and expression of angiopoietin-1 (Ang-1), angiopoietin-2 (Ang-2), Tie2, and VEGF messenger RNA (mRNA) was examined by real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR). Expression pattern of each gene in HCC and NCL tissue specimens was compared and the potential role and interaction in angiogenesis of HCC were analyzed. Genes' expression level and its relationship with tumor's clinicopathological parameters were also investigated. Immunohistochemical staining of CD34 was performed to determine the microvessel density (MVD) and Ang-2/Ang-1 ratio was calculated. Relationships between Ang-2/Ang-1 ratio, VEGF and MVD and clinicopathological features were also tested so as to evaluate their significance in the progression of HCC.

RESULTS: Ang-2 and VEGF mRNAs in HCC were significantly higher than those in NCL tissue ($P < 0.05$), whereas the Ang-1 and Tie2 mRNAs showed no statistical significance ($P > 0.05$), though slightly lower level of Ang-1 mRNA in HCC was observed. Ang-2/Ang-1 ratio and VEGF were both positively correlated to MVD. The Ang-2/Ang-1 ratio, Ang-2 and VEGF were all associated with tumor's clinicopathological parameters ($P < 0.05$) except for histological grades ($P > 0.05$). Ang-1 and Tie2 levels in different clinicopathological groups were not significantly different ($P > 0.05$).

CONCLUSION: Dominant Ang-2 expression against

Ang-1 through Tie2 receptor in the presence of VEGF plays a critical role in initiating early neovascularization and transformation of noncancerous liver to hepatocellular carcinoma. Its consequently constant operation in formed HCC induces further angiogenesis and progression of HCC.

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Key words: Hepatocellular carcinoma; Vascular endothelial growth factor; Angiopoietin; Tie2; Angiogenesis; Neovascularization

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<http://www.wjgnet.com/1007-9327/12/4241.asp>

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most malignant tumors in the tropics and the Far East, including China. It is the fourth most common cause of cancer death and accounts for 53% of all liver cancer deaths worldwide^[1]. HCC is also a hypervascular carcinoma. It is believed that angiogenesis contributes to its malignant biological characteristics such as invasion and high rates of recurrence and metastasis^[2-4]. Angiogenesis is a neovascularization process during which endothelial cells of the pre-existing capillaries proliferate and migrate to form new vascular tips or so-called "vascular sprouts" or "endothelial buds"^[5]. The growth, invasion and metastasis of malignant tumors depend on the process of angiogenesis. There is evidence that solid tumors do not grow beyond 2-3 mm³ in volume when vascular sprouts are blocked^[6].

Angiogenesis is a very complicated network which is closely regulated by many angiogenic factors. Vascular endothelial growth factor (VEGF) and angiopoietin are the two most important regulators. The latter is a novel family of angiogenic factors including Ang-1, Ang-2, Ang-3, and Ang-4, which have been isolated and identified as a group of ligands of the tyrosine kinase Tie2 receptor^[7,8]. Ang-1 and Ang-2 have been reported as the most potent regulators for neovascularization^[9] and are the activator and an-

tagonist of Tie2 receptor, where binding of Ang-1 causes autophosphorylation of Tie2 whereas Ang-2 binding suppresses the autophosphorylation^[9,10]. It was reported that proper regulation of tyrosine kinase Tie2 is absolutely required for normal vascular development, apparently by regulating vascular remodeling and maturation^[11]. Ang-1 helps to maintain and stabilize maturation vessels by promoting interaction between endothelial cells and support cells, such as pericytes. Knockout mice deficient in Ang-1 develop severe vascular defect and die in uterus, similar to Tie2 deficient mice^[12]. Ang-2 acts as an alternative ligand for Tie2 and binds to Tie2 with similar affinity, but competitively antagonizes Ang-1 effects with blockage of Tie2 phosphorylation and activation. Functionally, transgenic mice over-expressing Ang-2 show even more severe vascular defects as the Ang-1 or Tie2 deficient mice^[13]. In the presence of VEGF, vessel destabilization caused by Ang-2 has been hypothesized to induce angiogenic response, whereas in the absence of VEGF, Ang-2 leads to vessel regression^[13,14].

Although the exact role of angiotensin/Tie2 system remains enigmatic, there is evidence that this system in the presence of VEGF is important for the initiation of angiogenesis and vascular sprouting in tumors^[15]. It was recently reported that VEGF and angiotensin/Tie2 system play a key role in the transformation of normal lung to non-small cell lung carcinoma^[16]. However, their exact role in the initiation and development of HCC is still unclear. In this study, we investigated the expression of Ang-1, Ang-2, Tie2 and VEGF by real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR). Thirty-eight specimens of HCC and noncancerous liver (NCL) tissues were examined in an attempt to definite their role.

MATERIALS AND METHODS

Patients and specimens

Fresh surgically resected specimens of HCC and noncancerous liver tissue were obtained from 38 patients with HCC who underwent partial hepatectomy in the Department of General Surgery of Zhongnan Hospital, Wuhan University (Wuhan, China) from 2003 to 2005. Demographic data of all patients are shown in Table 1. Thirty-four patients suffered from hepatitis and liver cirrhosis at different extent. Diagnosis of all patients was confirmed by histological examination after operation, and the noncancerous liver tissue was resected. Fully informed consent was obtained from all patients, and the study was performed in accordance with the guidelines of the Helsinki Declaration of 1975 amended in 1983. Those who accepted any therapy or accompanied any other severe complications and those with metastatic liver tumor were excluded. Some samples were frozen in liquid nitrogen immediately after resection and the remaining were fixed in 10% formalin, embedded in paraffin and stained with hematoxylin-eosin for pathological examination. Histological grade was classified according to the criteria of Edmondson and Steiner.

Real-time semiquantitative RT-PCR assay

Total RNA was extracted with Trizol reagent (Promega,

Table 1 Demographic data and clinicopathological features in 38 cases of HCC

No.	Sex	Age	Tumor size (cm)	Tumor capsule	Metastasis	Portal vein invasion	Histological grade	Liver cirrhosis
1	M	51	< 5	+	-	-	G2	-
2	M	49	< 5	+	-	-	G3	+
3	M	31	> 10	-	-	-	G2	+
4	M	59	5-10	+	-	-	G3	+
5	F	55	> 10	-	-	-	G1	+
6	M	65	> 10	-	-	+	G3	+
7	M	56	> 10	-	+	+	G2	+
8	M	51	> 10	-	-	+	G3	+
9	F	49	< 5	+	-	-	G1	-
10	M	50	5-10	-	-	-	G2	+
11	M	53	> 10	-	-	+	G2	+
12	M	43	< 5	+	-	-	G3	+
13	M	36	5-10	+	-	-	G2	+
14	F	46	5-10	-	-	-	G1	+
15	F	45	5-10	-	-	-	G1	+
16	M	52	5-10	-	-	-	G2	+
17	F	50	5-10	-	-	-	G1	+
18	F	52	< 5	+	-	-	G1	+
19	F	61	> 10	-	+	+	G1	+
20	M	36	5-10	-	-	-	G2	+
21	M	46	< 5	+	-	-	G2	+
22	M	48	> 10	-	-	+	G2	+
23	F	63	< 5	+	-	-	G1	+
24	M	35	5-10	+	-	-	G2	-
25	M	74	> 10	-	+	+	G3	+
26	F	55	5-10	-	-	-	G1	+
27	M	59	5-10	-	-	-	G2	+
28	M	30	< 5	+	-	-	G3	+
29	F	56	5-10	-	-	-	G1	+
30	M	60	5-10	-	-	-	G2	+
31	F	65	< 5	+	-	-	G1	+
32	M	59	> 10	-	+	+	G2	+
33	M	61	> 10	-	+	+	G2	+
34	M	55	< 5	-	-	-	G2	-
35	M	48	5-10	+	-	-	G3	+
36	M	49	> 10	-	-	+	G3	+
37	F	50	5-10	-	-	-	G1	+
38	M	58	> 10	-	+	+	G3	+

USA) following the manufacturer's instructions and quantitated by absorbance analysis at 260 nm. First-strand cDNA was synthesized using first standard buffer, dNTP mixture containing each deoxynucleotide triphosphate base dithiothreitol and moloney murine leukemia virus RT (GIBCO, BRIL) as previously described^[17]. Four-fold dilution of the products was used for PCR in a Rotor-Gene2000 real-time PCR machine (Corbett Research, Australia). Primers of each gene are shown in Table 2 as previously described^[10]. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used as an internal standard. Reverse transcription was performed at 50°C for 30 min. The samples were subjected to PCR analysis using the following cycling parameters: at 95°C for 10 min, then at 95°C for 15 s and at 60°C for 1 min for 40 cycles. Negative controls (cDNA-free solutions) were included in each reaction. Standard reaction curve was analyzed by Rotor-Gene 5.0 (Corbett Research)

Table 2 Primer sequences of each gene for real-time RT-PCR assay

Target gene	Sense	Antisense
Ang-1	ACTGT GCAGA TGTAT ATCAA GC	GTGGA ATCTG TCATA CTGTG AA
Ang-2	GGAAG ACAAG CACAT CATCC	AGTAA GCCTG ATTCC CTTCC
Tie2	TCTGT GCTGT TCCTT CTTGC	CTTGA GTAAC TTCCA GCGGA
VEGF	AGCTA CTGCC ATCCA ATCGC	GGGCG AATCC AATTC CAAGA G
G3PDH	GTCAA CGGAT TTGGT CTGTA TT	AGTCT TCTGG GTGGC AGTGA T

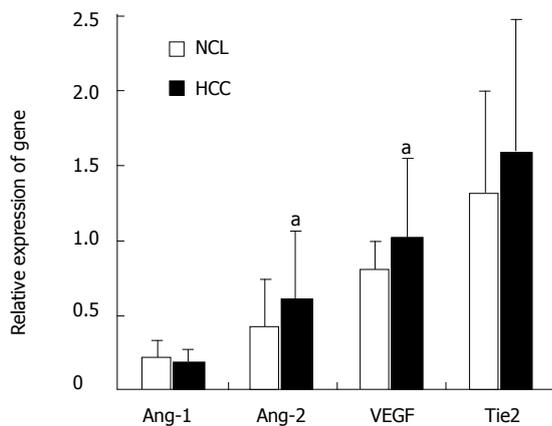


Figure 1 Expression of Ang-1, Ang-2, Tie2 and VEGF in HCC and NCL. HCC: hepatocellular carcinoma; NCL: noncancerous liver tissue. ^a $P < 0.05$ vs NCL.

software and relative quantity according to standard reaction curve (R_v) was calculated according to the formula $R_v = R_{Gene}/R_{G3PDH}$ by computer.

Immunohistochemical assessment of vessel density

Paraffin-embedded tissues were sectioned (4 μ m). The slides were deparaffined as usual, washed with tris buffered saline (TBS), and then incubated with 10% normal goat serum (Zhongshan Bio. CA). The sections were incubated with appropriately diluted (1:10) rat anti-human CD34 monoclonal antibody (Santa Cruz Biotechnology, CA) for 24 h at 4°C. Primary antibody was removed and washed with TBS, goat-anti-rat IgG peroxidase (Zhongshan Bio. CA) was then added. Finally the slices were stained as usual with haematoxylin and washed with distilled water. Quantification of blood vessels was carried out as previously described^[18]. Any brown-stained endothelial cell cluster distinct from adjacent microvessels, tumor cells, or other stromal cells was considered as a single countable microvessel. The most vascular areas of tumors were identified on a low-power field ($\times 100$), and vessels were counted in five high-power fields ($\times 200$). The data were presented as mean \pm SD. The process was performed by special pathologists in a blind manner.

Statistical analysis

Data were analyzed for significance with unpaired *t* test

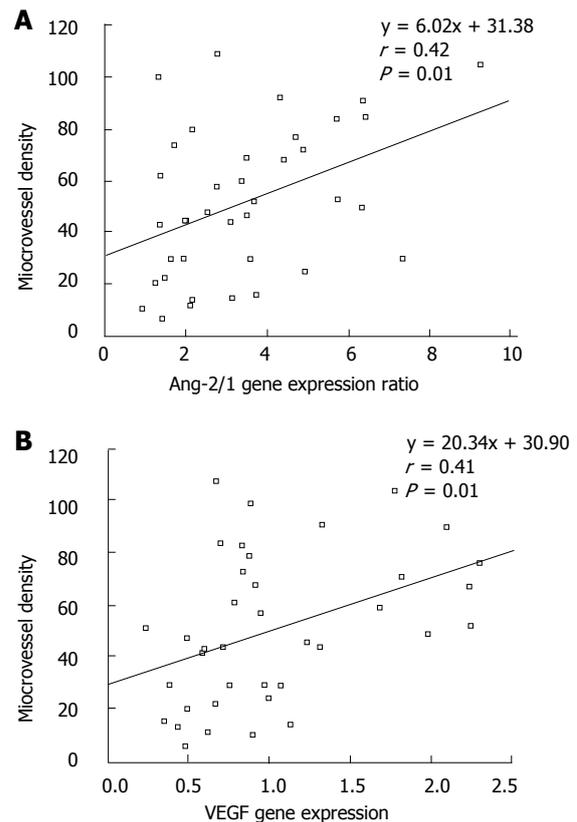


Figure 2 Relationship between Ang-2/Ang-1 ratio and microvessel density (A) and between vascular endothelial growth factor (VEGF) and microvessel density (B).

and ANOVA test. Statistical software SPSS 11.5 was used in the analysis. $P < 0.05$ was considered statistically significant.

RESULTS

Expression of Ang-1, Ang-2, Tie2 and VEGF in NCL and HCC

The expression of Ang-2 and VEGF mRNA was significantly higher in HCC than in the NCL tissue ($P < 0.05$), but the Ang-1 and Tie2 mRNA showed no statistical significance ($P > 0.05$), though slightly lower level of Ang-1 in HCC was observed (Figure 1).

Correlation between Ang-2/Ang-1 ratio, VEGF and MVD

The expression of CD34 in NCL tissue was poor or even negative, but strong in HCC. Microvessel counting revealed that MVD was higher in HCC than in NCL. Because the balance between Ang-1 and Ang-2 mainly defines Tie2 signal, we determined the ratio of Ang-2 to Ang-1 and evaluated its correlation with MVD. VEGF was also involved. The Ang-2/Ang-1 ratio in HCC was positively related to MVD ($P = 0.01$, $r = 0.42$) and VEGF ($P = 0.01$, $r = 0.41$) (Figure 2).

Relationship between gene expression pattern and clinicopathological parameters

The values of Ang-1, Ang-2, Tie2, VEGF and Ang-2/Ang-1 ratio were classified according to HCC clinicopathological parameters including tumor size, tumor capsule, portal vein invasion, distal metastasis and histological

Table 3 Relation between gene expression pattern and clinicopathological parameters (mean \pm SD)

Clinical parameters	Ang-2	Ang-1	Ang-2/1	Tie2	VEGF
Tumor size (cm)					
< 5 (n = 10)	0.267 \pm 0.113	0.148 \pm 0.089	2.175 \pm 1.025	1.686 \pm 0.550	0.552 \pm 0.205
\geq 5 (n = 28)	0.748 \pm 0.422	0.212 \pm 0.082	3.880 \pm 2.067	1.570 \pm 0.990	1.213 \pm 0.537
Histological grade					
G1 (n = 12)	0.509 \pm 0.320	0.168 \pm 0.064	3.106 \pm 1.409	1.511 \pm 0.574	0.916 \pm 0.497
G2 (n = 16)	0.633 \pm 0.395	0.194 \pm 0.081	3.543 \pm 1.925	1.470 \pm 0.726	1.095 \pm 0.604
G3 (n = 10)	0.737 \pm 0.512	0.234 \pm 0.101	3.645 \pm 2.470	1.921 \pm 0.138	1.097 \pm 0.585
Portal vein invasion					
positive (n = 11)	1.152 \pm 0.376	0.232 \pm 0.053	5.096 \pm 1.691	1.880 \pm 1.494	1.791 \pm 0.423
negative (n = 27)	0.405 \pm 0.163	0.180 \pm 0.092	2.753 \pm 1.631	1.488 \pm 0.500	0.732 \pm 0.254
Metastasis					
positive (n = 6)	1.320 \pm 0.366	0.235 \pm 0.061	5.912 \pm 1.847	2.262 \pm 1.734	2.007 \pm 0.214
negative (n = 32)	0.490 \pm 0.271	0.187 \pm 0.088	2.967 \pm 1.600	1.148 \pm 0.619	0.857 \pm 0.415
Tumor capsule					
positive (n = 13)	0.308 \pm 0.135	0.178 \pm 0.112	2.078 \pm 0.887	1.733 \pm 0.424	0.579 \pm 0.198
negative (n = 25)	0.783 \pm 0.429	0.203 \pm 0.068	4.135 \pm 1.998	1.529 \pm 1.056	1.278 \pm 0.558

grades. Ang-2, VEGF and Ang-2/Ang-1 ratio were all associated with the above clinicopathological parameters ($P < 0.05$) except for histological grades ($P > 0.05$), whereas Ang-1 and Tie2 showed no relation with any of the clinicopathological parameters ($P > 0.05$) (Table 3).

DISCUSSION

VEGF and angiopoietin are two of the most important regulators for neovascularization. The former is the most potent angiogenic factor that promotes endothelial proliferation and increases vascular permeability by binding to its specific receptors in endothelial cells such as Flt-1, KDR/Flk-1 and Flt-4^[19]. Animal models and *in vitro* experiments have shown that Ang-1, Ang-2 and Tie2 in association with VEGF constitute a system that regulates vascular quiescence and endothelial plasticity, through which a balanced state of vascular maturity and development of complex vascular networks can be achieved^[20]. In the current study we investigated the expression of Ang-1, Ang-2, Tie2 and VEGF mRNA in HCC and noncancerous liver in an attempt to definite their exact role in carcinogenesis and progression of HCC *via* angiogenesis. To avoid possible influence of indefinite borderline carcinoma invasion, the noncancerous liver tissue was obtained from HCC position as far as possible during surgery. Ang-2, as an actively angiogenic factor in the presence of VEGF, is little expressed in physiological condition but highly expressed in several actively neovascularized organs such as endometrium, ovary, placenta *etc*^[9,20-22]. This factor is up-regulated in many carcinomas, such as gastric, ovarian, colorectal and breast cancer, *etc*^[23-26]. In this study, Ang-2 mRNA was significantly up-regulated in HCC compared with the non-tumorous liver tissue, which is consistent with previous reports^[2,27] and has been regarded as a contributor to recurrence, metastasis and poor prognosis of HCC. Besides, high expression of Ang-2 and low expression Ang-1 were found in HCC in comparison with NCL, indicating that they play a key role in the carcinogenesis and progression of HCC *via* angiogenesis. Tumorous angiogenesis is very

different from the physiological process^[28]. During this procession, vascular quiescence and stabilization are mediated by Ang-1, Ang-2 and Tie2 system. Therefore, the pathologic state of imbalanced Ang-2/Ang-1 ratio in the presence of VEGF plays a critical role in the transformation of noncancerous liver to liver cancer by initiating early neovascularization. Vajkoczy *et al*^[29] reported that tumors in their very early stage are initiated by host vessels via VEGF, VEGF receptor-2 and Ang-2. Wong *et al*^[16] also reported that angiopoietins, tie2 and VEGF are differentially expressed in the transformation of normal lung to non-small cell lung carcinomas.

CD34 protein, an endothelial-specific marker, was immunohistochemically stained and MVD was determined in HCC in our study. Ang-2/Ang-1 ratio was calculated and statistical analysis showed that Ang-2/1 ratio was positively correlated to MVD, and VEGF, but such relation was not found between Ang-1, Tie2 and MVD. Ang-2/Ang-1 ratio, Ang-2 and VEGF are all associated with tumor's clinicopathological characteristics such as tumor size, tumor capsule, portal vein invasion and metastasis, indicating that VEGF and angiopoietin/Tie2 system contributes greatly to the progress of HCC by modulating angiogenesis. Continuous growth of carcinoma induces hypoxia and necrosis in central portion, thus reversely up-regulating VEGF^[30]. In the presence of Ang-2, tumor and invasion angiogenesis are greatly accelerated. A study^[31] on C6 glioma showed that Ang-2 expresses strongly whereas VEGF expresses poorly when the tumor reaches 1mm in diameter, and when the tumor reaches more than 2 mm in diameter necrosis occurs in the central portion, both Ang-2 and VEGF are strongly expressed which induces intensive angiogenesis. Interestingly, in the current study Ang-1, Ang-2, Tie2, VEGF and Ang-2/Ang-1 ratio were all not statistically associated with histological grades of HCC, which is similar to a previous report on ovarian cancer^[32].

In conclusion, expression of Ang-2 against Ang-1 through the Tie2 receptor in the presence of VEGF plays a critical role in initiating early neovascularization and in-

duces transformation of noncancerous liver to HCC. The consequently constant immature neovascularization in HCC further promotes angiogenesis and progression of tumors.

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RAPID COMMUNICATION

Genetic instability of BRCA1 gene at locus D17S855 is related to clinicopathological behaviors of gastric cancer from Chinese population

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Abstract

AIM: To investigate genetic instability of gene BRCA1 at locus D17S855, and their relationship with clinicopathological characteristics of gastric cancer in Chinese population.

METHODS: Microsatellite instability (MSI) and loss of heterozygosity (LOH) of gene BRCA1 at locus D17S855 were compared between 37 samples of gastric cancer and corresponding non-cancerous gastric tissue.

RESULTS: MSI at locus D17S855 was positive in 7 of 37 samples of gastric cancer (18.95%). MSI had a close relationship with TNM staging but no relation with lymph node metastasis, histological type or tumor differentiation. MSI positive frequency in TNM I + II (31.58%, 6/19) was much higher than that in TNM III + IV (5.56%, 1/18), ($P < 0.05$). LOH positive rate was 18.92% (7/37). LOH had no relationship to histological type, tumor differentiation or lymph node metastasis, but LOH positive rate in TNM III + IV was 33.33% (6/18), much higher than that in TNM I + II (5.26%, 1/19), ($P < 0.05$). BRCA1 protein was expressed in 14 of 37 samples of gastric cancer. The positive rates of BRCA1 protein in TNM I + II and TNM III + IV were 57.89% and 16.67%, respectively, ($P < 0.05$). The positive rate of BRCA1 protein was 77.78% in high differentiation samples, 30.77% in middle differentiation and 12.50% in lower differentiation samples, ($P < 0.05$).

CONCLUSION: MSI of BRCA1 gene could be used as a molecular marker in early phases of sporadic gastric cancer in Chinese population. LOH occurs at later period of gastric cancer, therefore, it could be used as prognostic factor.

INTRODUCTION

Gastric cancer is one of the most common cancers worldwide, and growing evidence suggests that accumulation of multiple alterations such as activation of proto-oncogenes and inactivation of tumor suppressor genes is responsible for the development and progression of gastric cancer. Genetic instability of oncogenes such as microsatellite instability (MSI) and loss of heterozygosity (LOH) is probably associated with mutations in genes responsible for tumor-genesis, and they play important roles in tumor clinical pathology^[1-4]. The studies of MSI and LOH of gastric cancer have been focused on genetic instability of P53, P16 and FHIT, but studies of gene BRCA1 gene are very few. The gene BRCA1 is located in the chromosomal region 17q21. Many studies have reported that BRCA1 is a tumor suppressor gene of breast and ovarian tumors, but only few studies have been done on Chinese gastric cancer^[5-7]. The present study was undertaken to investigate MSI and LOH of gene BRCA1 at locus D17S855 in Chinese gastric cancer, their influence on the expression of BRCA1 protein, and their relationship with clinical pathological characteristics of gastric cancer.

MATERIALS AND METHODS

Tumor samples and DNA extraction

A total of 37 formalin-fixed, paraffin-embedded tumor samples and corresponding non-tumor samples from the same sporadic gastric patients were collected. These samples included 30 cases of tubular adenocarcinoma and 7 cases of mucoid adenocarcinoma. All the samples were sectioned into 10 μ m sections, and tumor samples contained at least 90% tumor cells. Genomic DNAs

from tumor and normal components were extracted by proteinase K and phenol-chloroform extraction methods.

Polymerase Chain Reaction (PCR)

Primers of D17S855 locus were: 5'-GGA TGG CCT TTT AGA AAG TGG- 3' and 5'-ACA CAG ACT TGT CCT ACT GCC -3' (Bioasia Shanghai)^[8]. Polymerase chain reaction (PCR) was carried out in 50 μ L reaction mixtures containing 1.0 mmol/L MgCl₂, 10 \times Buffer 5 μ L, 200 μ mol/L dNTP, Taq polymerase 2 units and primers 50 μ mol/L. PCR was carried out for one cycle of 94°C for 4 min followed by 35 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, followed by a final extension for 10 min at 72°C. The PCR productions were detected by 2% agarose gel containing ethidium bromide, underwent electrophoresis for 30 min at 15 V/cm, and were examined under ultraviolet light.

Single strand conformation polymorphism analysis of MSI and LOH

The PCR production and an equal volume of denaturing stop solution (98% deionised formamide, 0.05% bromophenol blue and 0.05% xylene cyanol) were heated for 10 min and then were rapidly cooled on ice. Electrophoresis was carried out on 8% polyacrylamide gels at 100 V for 4 h. After electrophoresis, the gels were ordinarily silver-stained. To detect MSI and LOH, the PCR productions of normal and tumor DNA from the same patients were run in adjacent lanes. MSI was positive as either tumor samples added an allele band or moved as compared with normal tissue. LOH was positive as tumor samples lacked an allele band as compared with normal tissue.

Immunohistochemistry staining

Immunohistochemistry for BRCA1 protein was performed on 5 μ m sections from paraffin-embedded tumor tissue which was dewaxed in xylene and dehydrated in a graded ethanol series. The sections were immersed in 3% hydrogen peroxidase for 10 min to block endogenous peroxidase activity, and then rinsed in water. Thereafter, the sections were heated in a microwave for 5 min at 100°C to retrieve antigen. The sections were incubated with primary antibody BRCA1 (Santa Cruz Biotechnology, USA), then the sections were allowed to react by standard Envision method using secondary antibody (DAKO, Denmark). The binding was visualized by DAB, and then the samples were counterstained with hematoxylin.

Imaging analysis and data collection

After immunohistochemistry staining, sections were analyzed by Leica-Qwin computer imaging techniques. We selected twenty continuous high microscopical views that did not overlap. Then we tested the gray-value of background and named as GA. The gray-value of BRCA1 positive granules was named as G_a and area density of BRCA1 positive cells in the view area was named as A_{Aa}. We used Excel function to compute the value of PU (positive unit) which represented expression intensity of

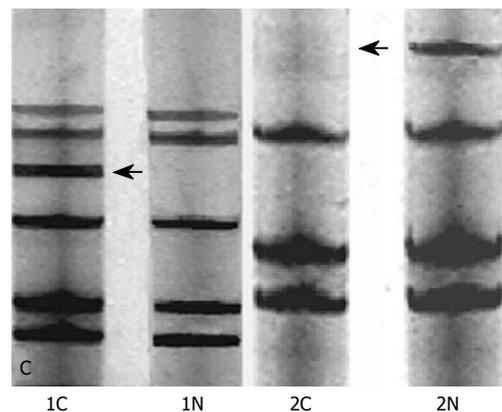


Figure 1 Polyacrylamide gel electrophoresis (PAGE) of BRCA1 gene at D17S855 locus Compared with normal tissue (1N), MSI was positive as added an allele band (\uparrow) in tumor tissue (1C). Compared with normal tissue (2N), LOH was positive as lacked an allele band (\uparrow) in tumor tissue (2C).

BRCA1 protein in gastric cancer cell. The highest gray value was 255 in Leica-Qwin system.

Statistical analysis

Statistical analysis was performed using analysis of variance (AVONA) and *t*-test. A *P* value of < 0.05 was considered significant difference.

$$PU = \frac{G_a - G_A}{(1 - A_{Aa}) \times 255} \times 100$$

RESULTS

Single strand conformation polymorphism analysis (PCR-SSCP) of MSI and LOH

In our experiment, 37 paired normal/tumor DNAs were successfully amplified by PCR method, and were tested by 8% polyacrylamide gels. MSI was positive as added an allele band in tumor tissue as compared with normal tissue; LOH was positive as lacked an allele band in tumor tissue as compared with normal tissue (Figure 1).

Of locus D17S855 at gene BRCA1, the positive frequency of MSI in 37 cases of gastric cancer was 18.92% (7/37) (Table 1). MSI of BRCA1 gene was significantly correlated with clinical TNM staging and degree of tumor differentiation; however, there was no significant difference between MSI-positive and MSI-negative cases in tumor histological type or lymph node metastasis. In tumor node metastasis (TNM) staging, the positive frequency of MSI in stage TNM I + II (31.58%) was more than that in stage TNM III + IV (5.56, *P* < 0.05), and the frequency of LOH-positive of 37 cases of gastric cancer was 18.92% (7/37) (Table 2). LOH of BRCA1 gene was significantly correlated with clinical TNM staging; however, there was no significant difference between LOH-positive and LOH-negative cases in tumor histological type, tumor differentiation degree or lymph node metastasis. In tumor node metastasis (TNM) staging, the positive frequency of LOH in stage TNM III + IV (33.33%) was higher than that in stage TNM I + II (5.26%, *P* < 0.05).

Table 1 Relationship between hereditary instability of BRCA1 gene at locus D17S855 and clinicopathological parameters of gastric cancer

Clinicopathological parameters	Case (n)	MSI positive rates (%)	LOH positive rates (%)	BRCA1 positive rates (%)	BRCA1 expression (PU Mean ± S)
Histology	37	7 (18.92%)	7 (18.92%)	14 (37.84%)	33.56 ± 2.23
Tubuladenoma	30	6 (20.00%)	6 (20.00%)	12 (40.00%)	32.87 ± 2.93
High differentiation	9	4 (44.44%) 1 (7.69%)	1 (11.11%)	7 (77.78%) ^a	34.03 ± 1.79
Middle differentiation	13	1 (12.50%)	1 (7.69%)	4 (30.77%)	33.49 ± 2.34
Low differentiation	8	1 (14.29%)	4 (50.00%)	1 (12.50%)	33.58 ± 1.67
Mucoadenoma	7		1 (14.29%)	2 (28.57%)	32.87 ± 2.59
Lymph metastasis					
No	17	5 (29.41%)	1 (5.88%)	9 (52.94%)	34.75 ± 1.78
Yes	20	2 (10.00%)	6 (30.00%)	5 (25.00%)	33.14 ± 2.14
TNM staging					
Stage I + II	19	6 (31.58%)	1 (5.26%)	11 (57.89%)	33.88 ± 2.38
Stage III + IV	18	1 (5.56%) ^a	6 (33.33%) ^a	3 (16.67%) ^a	33.23 ± 2.34

^a*P* < 0.05.

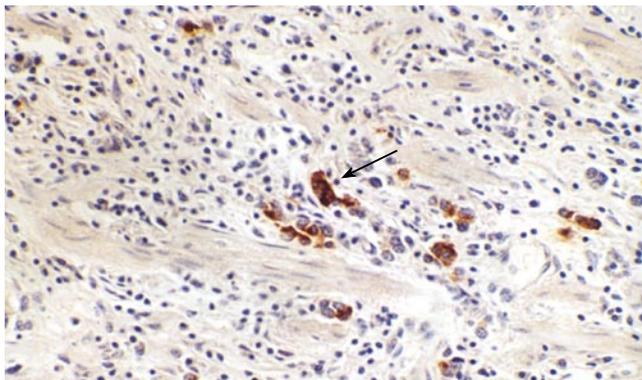


Figure 2 The expression of BRCA1 protein in gastric cancer. The brown-yellow granules of BRCA1 protein were mostly located in nucleolus. The cytoplasm and membrane of cells were also stained (HE stained†). ×100.

Immunohistochemistry analysis of BRCA1 protein

The expression of BRCA1 protein in gastric cancer was the brown-yellow granules, mostly located in nucleolus, and cytoplasm and membrane of cells were also stained (Figure 2). The frequency of BRCA1 protein-positive in 37 cases of gastric cancer was 37.84% (14/37) (Table 2). Expression of BRCA1 protein was significantly correlated with clinical TNM staging and tumor differentiation degree; however, expression of BRCA1 protein was not associated with tumor histological type or lymph node metastasis. The positive frequency of BRCA1 protein in TNM I + II (57.89%) was much higher than TNM III + IV stage (16.67%, *P* < 0.05), and BRCA1 positive-rate in well differentiation cases was higher than poor differentiation cases. The frequency of BRCA1 protein-positive decreased as tumor differentiation went down, 77.78% in high differentiation cases, 30.77% in middle differentiation cases, and 12.50% in low differentiation

Table 2 Relationship between MSI, LOH of BRCA1 gene at locus D17S855 and BRCA1 protein expression

Grouping	Cases	BRCA1 protein positive rates	BRCA1 expression (PU Mean ± S)
MSI positive group	7	85.71% (6/7)	34.05 ± 1.91
MSI negative group	30	26.67% (8/30) ^a	32.59 ± 2.23
LOH positive group	7	28.57% (2/7)	32.87 ± 2.35
LOH negative group	30	40.00% (12/30)	34.03 ± 2.66

^a*P* < 0.05.

cases (*P* < 0.05). There was no difference in BRCA1 protein expression intensity analyzed by computer imaging.

DISCUSSION

In 1994, the breast-cancer susceptibility gene, BRCA1, was identified by positional cloning; subsequently, this gene has been the subject of intensive research effort. BRCA1, located on chromosome 17q21 and encoding a tumor suppressor gene that functions, in part, as a caretaker gene in preserving chromosomal stability, is composed of 22 coding exons distributed over 100 kb of genomic DNA^[3]. This gene encodes 1863 amino acids, and more than 200 different germline mutations associated with cancer susceptibility have been identified. Cell cycle checkpoints play an essential role in cell survival by preventing the propagation of DNA damage through cell cycle progression before DNA repair. Recent studies have demonstrated that both ATM and BRCA1 are required for effective S-phase and G2/M-phase checkpoints. Other work has indicated that BRCA1 regulates G2/M DNA damage induced checkpoints through its ability to activate Chk1 kinase and thereby induce signaling cascades downstream of Chk1. BRCA1 functions as a co-activator of p53-mediated gene transcription. Other studies have shown that overexpression of BRCA1 results in the transcriptional activation of GADD45 in a p53-dependent manner. As GADD45 has been implicated in G2/M checkpoints, BRCA1 may in part activate G2/M checkpoints by induction of GADD45 protein^[3,9,10].

The association of the BRCA1 gene with susceptibility to breast and ovarian cancer has been strongly demonstrated, and locus D17S855 was found as one of the best candidate loci to detect tumor suppressor genes^[10]. Mori *et al* found that BRCA1 might play an important role in the development of esophagus cancer with high frequent LOH^[9]. Therefore, locus D17S855 was used in this experiment to detect MSI and LOH in gastric cancer of Chinese population.

In this study, the frequency of MSI-positive in 37 cases of gastric cancer was 18.92%. MSI of BRCA1 gene was significantly correlated with clinical TNM staging and tumor differentiation degree; however, there was no significant difference between MSI-positive and MSI-negative cases in tumor histological type or lymph node metastasis. TNM staging represents the prognosis of tumor, and we found in the experiment that the frequency

of MSI in stage TNM I+ II was more than that in stage TNM III + IV. Therefore, as far as gene BRCA1 is concerned, high frequency of MSI endowed with a good prognosis. Furthermore, the positive frequency of MSI decreased as tumor differentiation went down, tumor differentiation correlated with the development and malignant degree of tumor. According to the experiment results, MSI of BRCA1 was an early period molecule marker of sporadic gastric cancer. Candusso *et al* found that LOH often occurred in the later stage of cancer, mostly with lymph metastasis^[10]. The positive frequency of LOH in 37 cases of gastric cancer in our study was 18.92%, and the positive frequency of LOH in stage TNM III + IV was higher than that in stage TNM I + II. The results suggested that LOH of BRCA1 gene might occur in the later stage of gastric cancer and could be a marker that predicts prognosis of gastric cancer.

The positive frequency of BRCA1 protein in MSI positive group was higher than MSI negative group, but there was no significant difference between LOH positive and negative groups. Expression of BRCA1 protein was significantly correlated with clinical TNM staging and tumor differentiation degree; however, expression of BRCA1 protein was not associated with tumor histological type or lymph node metastasis. The frequency of BRCA1 protein-positive decreased as tumor differentiation went down. All these data suggested that BRCA1 gene might restrain gastric cancer from developing further.

In conclusion, MSI of BRCA1 is an early event in development of sporadic gastric cancer in Chinese population and LOH of BRCA1 gene occurs in later stage, therefore BRCA1 gene might be a suppressor gene for gastric cancer.

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CASE REPORT

Multiple von Meyenburg complexes mimicking diffuse liver metastases from esophageal squamous cell carcinoma

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Abstract

Von Meyenburg complexes are benign liver lesions consisting of adenomatous bile duct proliferates. We present two patients suffering from esophageal cancer accompanied by the occurrence of von Meyenburg complexes. Preoperative computerized tomography (CT) of the liver had not shown these lesions. In one of the patients, diffuse nodular manifestation was found in both liver lobes, mimicking diffuse hepatic metastases. Intraoperative frozen section revealed the benign nature of the lesions in both cases. The patients underwent esophageal resection without complications. To the best of our knowledge, the coincidence of von Meyenburg complexes and esophageal cancer has never been reported before. This uncommon entity should be taken into consideration as a differential diagnosis of liver lesions in malignancies. It underlines the importance of intraoperative frozen section for liver lesions of unknown origin.

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Key words: Von Meyenburg complexes; Bile duct hamartomas; Esophageal carcinoma; Liver metastasis

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INTRODUCTION

Von Meyenburg complexes are benign liver lesions con-

sisting of dilated bile duct structures with a surrounding fibrous stroma. Their incidence is age-dependent and they are observed about 1% in children and 5%-6% in adults^[1]. Usually, these lesions do not have any pathological value and are incidental findings in asymptomatic patients^[1]. However, a malignant transformation and association with cholangiocarcinomas have been discussed, but not yet proven^[2,3]. As von Meyenburg complexes are small lesions, they are usually not detected on radiological examinations, including ultrasound, CT and magnetic resonance imaging (MRI)^[4-7].

We present herein two cases of preoperatively undetected von Meyenburg complexes associated with primary esophageal cancer and review the present literature concerning these lesions.

CASE REPORT

Patient 1 is a 66-year-old male suffering from an esophageal squamous cell carcinoma. Preoperatively, distant metastases were excluded by abdominal ultrasound, chest X-ray and high resolution CT-imaging of the thorax and liver (Figure 1). Surgical therapy with abdomino-thoracic resection of the esophagus was indicated.

Intraoperatively, abdominal exploration revealed multiple white nodular lesions in both lobes of the liver. With a diameter of 2-3 mm, they had the macroscopic aspect of diffuse liver metastases (Figure 2). To confirm the diagnosis of metastases, which would have excluded the patient from further curative resection of the primary esophageal carcinoma, a frozen section was performed. Surprisingly, histology showed benign microhamartomas, so-called von Meyenburg complexes (Figure 3). Because of the benign nature of the lesions, esophageal resection was performed as planned. The postoperative course was uneventful, liver function was normal and the patient recovered quickly.

Patient 2 is a 47-year-old male also suffering from an esophageal squamous cell carcinoma. Comparable to patient 1, the preoperative liver CT scan showed no pathological findings except for liver steatosis and a small cyst in segment IV/VIII.

Intraoperatively, we additionally found a suspicious liver lesion of 5 mm size in segment III. In contrast to the case described above, this was a solitary lesion, with the macroscopic aspect of a metastasis. However, frozen section revealed a sclerotic von Meyenburg complex. Consequently, the operation was performed as planned. No further complications occurred in the postoperative course.



Figure 1 Preoperative CT scan (patient 1). No parenchymal inhomogeneity or focal lesions can be detected.

DISCUSSION

Esophageal cancer is an important cause of cancer death, with an incidence of about 8-10/100 000 and 13 300 deaths in the United States in 2004^[8]. About 35% of all patients already suffer from liver metastasis by the time of diagnosis of the primary tumor. Since patients are excluded from potentially curative resection of the tumor when metastases are present, the diagnosis of liver metastases plays a crucial role for further treatment. The prognosis of these patients is also highly dependant on the respectability of the tumor.

Distant metastases, especially liver metastasis, can be diagnosed by CT scan or MRI with high sensitivity and specificity^[6]. Sensitivities of these diagnostic means range between 74% and 85%^[9]. In these series, almost all false-negative results occurred when lesions were less than 1.5 cm in diameter. Therefore, non-invasive detection of small metastases can be difficult or even impossible.

When suspicious lesions are found by CT scan, further differentiation is possible by additional MRI imaging^[5]. Differential diagnosis of liver metastases includes benign liver lesions, including hemangiomas, adenomas, von Meyenburg complexes or infectious lesions e.g. miliary tuberculosis^[5].

Bile duct hamartomas (von Meyenburg complexes) of the liver are usually detected during laparotomy or autopsy as an incidental finding. Multilocular occurrence is possible although they are rarely spread throughout the whole liver, as it was observed in our first patient. They may be found in normal liver tissue, but also in association with Caroli's syndrome, congenital hepatic fibrosis (CHF) or autosomal dominant polycystic kidney disease (ADPKD)^[10]. Histology of von Meyenburg complexes consists of a variable number of dilated small bile ducts, embedded in a fibrous, sometimes hyalinizing stroma (Figure 3).

If detected by CT scan or MRI, von Meyenburg complexes appear as small intrahepatic cystoid lesions. The lesions are frequently located adjacent to the portal veins, although the lesions can also be located everywhere else^[5]. However, it remains difficult to differentiate between metastases and benign liver lesions. Moreover, small liver



Figure 2 Intraoperative aspects (patient 1). Multiple small nodules are present in both lobes of the liver, mimicking diffuse metastatic lesions.

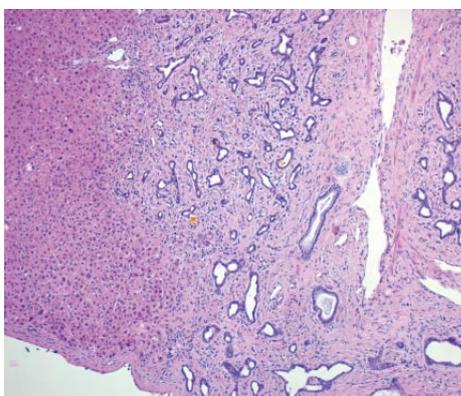


Figure 3 Histologic staining of a von Meyenburg complex (patient 1).

lesions with a diameter of less than 1.5 cm are often not detected by CT or MRI^[9].

Since the treatment of metastatic disease is completely different from resectable esophageal cancer, liver lesions need to be identified and characterized as early as possible. In our presented patients, the preoperative staging did not reveal any liver metastases. This underlines the importance of exact diagnostic measures in cases of unexpected intraoperative findings. Besides intraoperative ultrasound of the liver, frozen section is the gold standard for further differentiation of liver lesions of unknown origin.

Von Meyenburg complexes are defined as innocuous lesions. However, there are about 10 reported cases of neoplastic transformation of von Meyenburg complexes resulting in cholangiocarcinomas^[2,3].

In conclusion, von Meyenburg complexes are an important differential diagnosis of hepatic metastases. As the existence of liver metastases is crucial for therapeutic decision making in malignant diseases, this differential diagnosis must be carefully clarified. Since von Meyenburg complexes are usually less than 5 mm in size, they can escape preoperative radiologic diagnostics. The macroscopic appearance of von Meyenburg complexes can mimic liver metastasis as demonstrated in our reported patients. This underlines the importance of intraoperative frozen sections to make the correct diagnosis.

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Systemic lupus erythematosus following virological response to peginterferon alfa-2b in a transplanted patient with chronic hepatitis C recurrence

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Abstract

Autoimmune manifestations are common both in patients chronically infected by hepatitis C virus, and in patients transplanted for non-autoimmune diseases. A correlation between interferon based treatment and autoimmune diseases or the development of autoantibodies is well established in non-transplanted patients, but few data are available about transplanted patients. It is unclear whether interferon may increase the incidence of acute cellular rejection and there are few reports on the development of atypical autoimmune manifestations during post-liver transplantation interferon or pegylated interferon treatment. We describe a case of systemic lupus erythematosus following treatment with pegylated interferon alfa-2b in a transplanted patient with recurrence of chronic hepatitis C. Our experience suggest that pegylated interferon may induce autoimmune diseases in the immunosuppressed host, different from acute cellular rejection and call for a great attention to possible autoimmune disorders development during interferon based treatments in liver transplanted patients.

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Key words: Hepatitis C virus; Liver transplantation; Autoimmunity; Immunosuppression; Systemic lupus erythematosus

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INTRODUCTION

Autoimmune manifestations are common in patients chronically infected by hepatitis C virus (HCV)^[1]. On the other hand, tissue autoantibodies are common in liver recipients transplanted for non autoimmune diseases and may be associated with negative graft outcome^[2,3]. The safety and efficacy of interferon (IFNs) and the newest pegylated interferons (Peg-IFNs) for the treatment of recurrent hepatitis C in transplanted patients are still debated^[4,5]. In particular, it is unclear whether IFN may increase the incidence of acute cellular rejection (ACR) and there are no reports on the development of atypical autoimmune manifestations during post-liver transplantation (LT) IFN or Peg-IFN treatment.

We report a case of severe autoimmune disease, different from ACR, during treatment with Peg-IFN alfa-2b in a transplanted patient with recurrence of chronic hepatitis C (CHC).

CASE REPORT

A 55-year-old man, underwent LT in March 2001 for HCV genotype 1 liver related cirrhosis. Acute immunosuppressive (IS) schedule was cyclosporine, azathioprine (AZA) and steroids. According to the Transplantation Unit IS protocol, AZA and steroids were stopped 3 wk and 1 year after LT, respectively. Screening tests for LT revealed the presence of cryoglobulins with a cryocrite of 8% and antinuclear antibodies (ANA) at low titre (1/160) with homogeneous pattern. After LT, clinical outcome was regular until January 2002, when the patient showed a persistent mild increase of transaminases (ALT 115 U/L and AST 103 U/L) with high viral load (17.5 MEq/mL, Versant HCV-RNA 3.0 bDNA, Bayer). Liver histology showed mildly active chronic hepatitis with severe fibrosis, presence of lymphocytes and macrovesicular steatosis, sug-

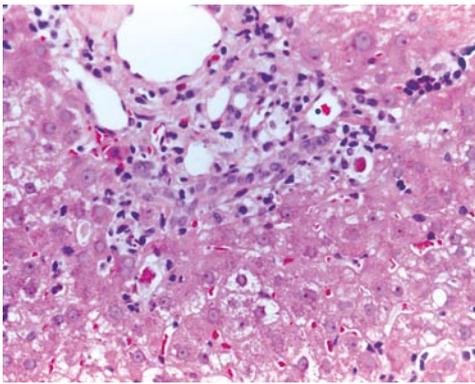


Figure 1 Liver histology before starting antiviral treatment.

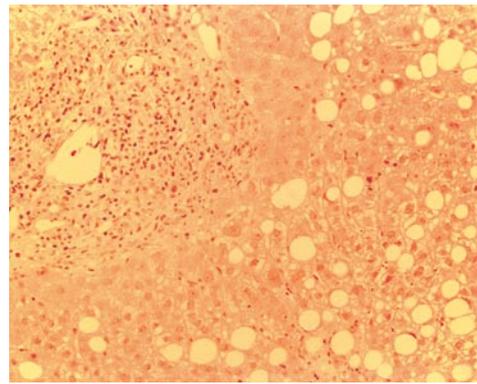


Figure 2 Liver histology after stopping antiviral treatment.

gestive of HCV recurrence (Figure 1).

In October 2002 the patient started a cycle of Peg-IFN alfa-2b (1.1 mcg/kg per week) and Ribavirin (6.4 mg/kg per day). After 4 wk of treatment transaminases were normal. HCV-RNA showed a 2 log fall (0.01 MEq/mL) at wk 12; became undetectable by branched DNA, but still positive by polymerase chain reaction (TMA test, Versant HCV-RNA, Bayer) at wk 24 and finally negative by PCR at wk 36.

At wk 44 the patient presented migrant arthritis and the following biochemical parameters: normal transaminases, CyA 240 ng/mL, increased gamma-glutamyltransferase (γ GT), alkaline phosphatase (ALP) and bilirubin (384 U/L, 690 U/L and 1.69 mg/dL, respectively), gamma-globulins 30%, Waaler-Rose 1/1280, ANA 1/640 and anti-DNA positive. No vascular or biliary complications were revealed by ultrasound and computed tomography, nor any signs of infectious diseases were present. Suspicion of an immune mediated manifestation, prednisone 10 mg/d was started. However, despite the presence of signs of autoimmunity we decided to complete the Peg-IFN cycle in consideration of the fact that we were almost at the end of the planned 48 wk of treatment with the patient responding to Peg-IFN.

At wk 48 the patient was asymptomatic, transaminases and bilirubin were normal, HCV-RNA negative by PCR, while ALP and γ GT were decreased (ALP 350 U/L and γ GT 94 U/L). Peg-IFN was stopped and steroids were maintained.

One month later, the patient developed pleuro-pericardial effusion and ascites. Liver function tests (LFTs) were normal, HCV-RNA was negative (PCR) and CyA within the therapeutic range; ANA was very high (1/1280) as well as perinuclear anti-neutrophil cytoplasmic antibodies (pANCA) (1:320). Therefore, our patient developed a syndrome characterised by high titre autoantibodies, migrant arthritis and serositis. Following the current criteria, the diagnosis of systemic lupus erythematosus could have been made^[6,7]. Prednisone was increased to 15 mg/d and diuretics introduced, resulting in the remission of liquid effusions and a progressive reduction of autoantibodies (ANA 1:160 and pANCA negative). We did not increase further the prednisone dosage in consideration of the possibility of an HCV re-activation in an already immunosuppressed patient and because of the initial clinical remission of autoimmune manifestations.

In March 2004, four months later, the patient presented an important increase of all LFT (GOT 257 U/L, GPT

197 U/L, ALP 438 U/L, γ GT 356 U/L, total bilirubin 20.35 mg/dL, with direct bilirubin 17.20 mg/dL) with normal eosinophils, serum HCV-RNA positive at low titre (2.96 MEq/mL). CyA was close to the lower limit of the therapeutic range (100 ng/mL). Liver biopsy revealed the presence of interface hepatitis, numerous rosettes, cholangitis with ductular proliferation and biliary regression (Figure 2). Histological findings were not suggestive for HCV recurrence and did not fulfil the standard criteria for acute or chronic rejection^[8]. Therefore, International Criteria for Autoimmune Hepatitis (AIH score)^[9] were applied, according to which the patient was categorized as positive for "probable AIH" (score + 11). Consequently, steroid treatment was increased and the patient was switched from cyclosporine to tacrolimus. Response to immunosuppressive treatment was slow but progressive until normalization of LFTs.

DISCUSSION

It is well known that treatment with IFN may cause autoimmune diseases or the development of autoantibodies in non transplanted patients^[10-13] while few data are available on transplanted ones. In particular, to the best of our knowledge, it has never been described the development of SLE after LT during interferon treatment. Only two cases of SLE have been described during IFN treatment in immunocompetent patients treated for HCV infection^[12]. In a series of 677 patients treated for HCV infection, 4% developed autoimmune diseases, the majority (62%) being represented by thyroid dysfunction; only 1 patient developed an SLE-like syndrome with an incidence of 0.15% in the study population^[14]. Alfa-IFN therapy has been moreover associated with the development of idiopathic thrombocytopenic purpura, myasthenia gravis, Addison's disease, diabetes, COELIAC disease, rheumatoid arthritis, primary biliary cirrhosis, polymyositis, psoriasis^[12,15-23].

Antiviral actions of alfa-IFN include the induction of several proteins, such as protein kinase (PKR) and 2',5'-oligoadenylate synthetase, important in viral dynamics^[24]. Moreover, IFNs induce the expression of MHC both on antigen presenting cells (APC) and hepatocytes, resulting in a virus-specific lysis of infected cells mediated by cytotoxic T-cell response^[25]. Thus, IFNs may lead to the development of autoimmune diseases by the up-regulation of MHC in both transplanted and non transplanted patients.

Moreover, the interaction of IFN activities in a particular pathway, such as in the immunosuppressed host, may lead to severe autoimmune manifestations that can compromise the graft survival. A relation between virological response and ACR was suggested in a recent study. Authors supposed that viral eradication improves microsomal function leading to a decrease of IS drugs levels^[4]. The same mechanism may play a role in the induction of other autoimmune diseases such as that occurred to our patient who developed an SLE. Furthermore, our patient was on treatment with calcineurin inhibitors that have been thought to predispose to autoimmunity by interfering with T cell maturation and developing autoaggressive T-cells clones^[26]. It is therefore possible that various mechanisms contribute to the development of autoimmune manifestations after liver transplantation.

In conclusion, the clinician should be aware of the possible development of autoimmune disorders, different from ACR, during interferon based treatment in LT patients, especially if signs of autoimmunity are present before starting IFN.

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CASE REPORT

A case of splenic abscess after radiofrequency ablation

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Abstract

Radiofrequency ablation (RFA) is an innovative technique used primarily for the palliative treatment of unresectable liver tumors. Its therapeutic indications however, have been expanded and now include various other organs and diseases. There is a paucity of data regarding technical details and complications of the use of RFA in the spleen. We report a case of partial splenectomy using radiofrequency ablation for splenic hydatid disease, complicated by an abscess formation.

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Key words: Radio frequency ablation; Partial splenectomy; Abscess; Hydatid disease

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INTRODUCTION

The spleen is an important component of the body's defenses against infection and the adverse consequences of its removal have become increasingly apparent over the last four to five decades. Death rates from overwhelming post splenectomy sepsis have been reported to be up to 600 times greater than those of the general population, with an estimated life time risk for post splenectomy sepsis of 5%. There is evidence that splenic reticuloendothelial function can be preserved by partial splenectomy, provided that at least 25% of splenic tissue is preserved^[1,2].

Partial splenectomy is not routinely practiced mainly because of the lack of vascular control to arrest bleeding^[3]. The most common indications included splenic trauma, staging and palliation of malignant diseases, inflammatory pseudotumors, hemangiomas, lipomas, hamartomas,

parasitic and non parasitic cysts and other benign conditions.

Radiofrequency ablation is a relatively new technique for local ablation of unresectable liver tumors^[4]. It has been successfully adapted as a treatment modality of primary and metastatic tumors of the liver, kidney, breast, and bone^[5]. Furthermore, the RF energy has been used successfully for the division of liver parenchyma^[6]. Very few clinical studies refer to the use of RF energy in the spleen. We report a case of partial splenectomy using RF energy complicated by an abscess formation.

CASE REPORT

A 52 year old man was admitted to our hospital with vague abdominal pain localized to the left upper quadrant of the abdomen. The patient's past medical history included an appendectomy performed 30 years ago and two operations for the removal of liver echinococcal cysts, performed 28 and 4 years ago. Physical examination and routine blood tests were unremarkable. The patient was found to be positive for anti-echinococcal antibodies, and thus two cycles of scolicedal agent were administered pre-operatively. The patient's radiographic work up, which included routine X-rays, an ultrasound and a computed tomography of the abdomen, revealed two hydatid cysts; one located at the lower splenic pole (9 cm × 10 cm × 8 cm) and the other located at the left pelvic floor (10 cm × 5 cm × 4 cm).

The patient underwent an exploratory laparotomy, through a midline incision. The abdominal cavity was entered with difficulty due to dense adhesions. The left colon was mobilized from the lateral abdominal wall, but the cyst was attached to the mesentery and subsequently its blood supply was sacrificed. Multiple erosions of the left colon were identified and subsequently a left colectomy and end colostomy were performed. The cyst was finally removed en block.

The spleen was surrounded by multiple dense adhesions and access to the splenic hilum was very difficult. However all the surrounding tissue was mobilized and pushed away from the cyst and the splenic pole. No vascular division took place. The cyst involved the lower pole parenchyma.

At this point division of the splenic parenchyma was decided in order to achieve en block removal of the hydatid cyst.

The Radionics Cooltip Radiofrequency System (Radionics/Tyco Hellas) was used to create a zone of desiccation between the cyst and the splenic



Figure 1 A zone of desiccation was created between the hydatid cyst and the splenic parenchyma using the RF electrode.

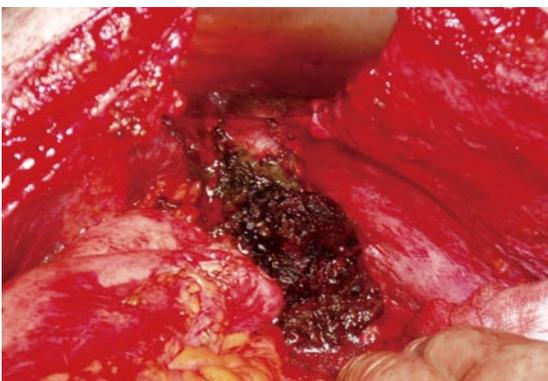


Figure 2 The splenic remnant after removal of the hydatid cyst and hemostasis using the radiofrequency energy.

parenchyma. A 3 cm Cooltip RF electrode was applied approximately 2 cm from the cyst wall and into the splenic parenchyma away from the hilum (Figure 1). The RF generator was turned on to the maximum power up to 140 W. Generator output, tissue impedance and electrode tip temperature were calculated, and measurement appeared on the generator's screen. Chilled saline was circulated by the perfusion pump through two coaxial cannulae in the probe during RF application to help prevent tissue boiling and cavitations immediately adjacent to the needle. The generator was activated to the maximum power and using the manual mode, the RF was applied until the impedance of the coagulated tissue reached a level twice as high as the initial measurement. Division of the splenic parenchyma was carried out using scissors. Residual bleeding was taken care of using RF again. The cyst was finally removed en block (Figure 2).

After copious irrigation and hemostasis the abdominal cavity was drained and closed in a standard fashion and the colostomy was matured. The pathology revealed macroscopically an intact hydatid cyst surrounded by approximately a 1 cm ring of coagulated splenic tissue.

For the first two post operative days, small amounts of dark bloody stained fluid was continuously flowing from the splenic bed drain. On the fourth postoperative day the patient became septic and he was transferred to the ICU in septic shock.

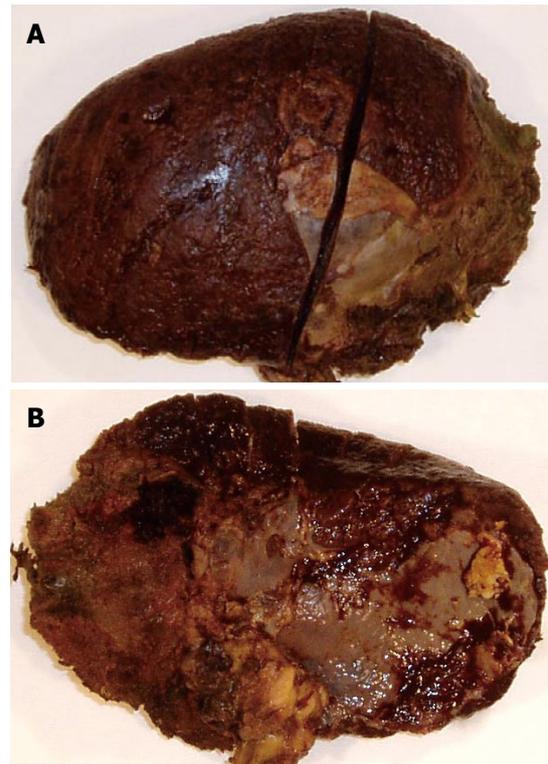


Figure 3 The splenic remnant was found to be infected with multiple microabscesses.

Urgent abdominal CT revealed the presence of atelectasis of the lower lobe of the left lung, with pleural effusion, gastric dilation and the presence of perisplenic abscess.

The patient was taken back to the operating room, and splenectomy was performed. The spleen was found to be infected and multiple abscesses surrounding the spleen were identified (Figure 3). The abdominal cavity was then washed out with copious amount of irrigation, and drains were placed again.

After 3 d in the Intensive Care Unit, the patient returned to the floor. Subsequently, the patient developed an enterocutaneous fistula, which was treated conservatively.

The pathology macroscopically revealed a splenic remnant that weighed 305 g and was 15 cm × 12 cm × 3 cm in size. A section of 1 cm of the parenchyma showed ash colored areas throughout the spleen, which became more prominent towards the lower pole.

Microscopically a zone of coagulative necrosis with deeper areas of liquefaction and hemorrhagic necrosis was identified. The rest of the spleen was characterized by changes in the architecture of the white pulp. The red pulp was characterized by intense dilation of the sinuses with infiltration of plasma cells and macrophages around the perivascular space throughout the spleen.

DISCUSSION

The incidence of septic complications, such as abscess formation, in RF assisted operations has been reported in various organs. In a recent review of 3670 patients

with liver tumors who underwent open, laparoscopic percutaneous or combined RFA the incidence of septic complications was 1.1%^[7]. Although there are no large series of RF assisted hepatectomies the reported incidence of an abscess formation after division of the liver parenchyma using RF energy remains low.

Septic complications resulting from the application of RF energy to the kidney, adrenals, breast and lungs have been documented to have an incidence of 1%. However all the reported studies have been conducted on a small series of patients^[5].

Predisposing factors for septic complications in RF assisted operations are diabetes mellitus, the existence of bilio-enteric anastomosis for the liver cases and a simultaneous "dirty" operation, like a colectomy, with the most common reported pathogen being the *Staphylococcus aureus*^[8].

In the presented case a colectomy was performed with a subsequent end colostomy due to multiple adhesions transforming a clean procedure into a "dirty one". In our opinion, that may be a predisposing factor for a septic complication following RF assisted partial splenectomy, though this has not been investigated in the literature in most series, since most of the studies refer to RF as a single procedure.

Intraoperative cultures of the abscess material obtained during the operation, grew an enteric flora *Enterococcus Faecium*, sensitive to a broad spectrum of antibiotics.

Experimental studies^[9,10] have established the safety and efficiency of RF energy to splenic tissue. Quanda Liu *et al*^[11] reported the first clinical series of nine patients who underwent RFA for hypersplenism in patients with liver cirrhosis, with no mortality and no morbidity, other than hydrothorax. However, in this study, the spleen was ablated but not divided. To the best of our knowledge, no data exists regarding the application of the RF energy for partial splenectomy to the splenic parenchyma other than few case reports with zero reported morbidity^[3,12].

We report a case of partial splenectomy using RF energy for hydatid cyst removal complicated by a splenic abscess.

Based on our experience of RF assisted liver resections, we applied maximum RF energy to create a zone of desiccation on the splenic parenchyma. However, the application of RF energy to the spleen is in many ways different than that performed on the liver

parenchyma. Perhaps the double blood supply to the liver and subsequently the hit sink phenomenon, makes the liver parenchyma less sensitive to the RF energy than that of the spleen. In our case the pathology of the splenic remnant revealed changes throughout the splenic parenchyma probably due to excessive applied energy.

In conclusion, we believe that RF assisted partial splenectomy should be performed with the least possible RF energy to achieve hemostasis.

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S- Editor Wang J E- Editor Liu Y

Primary melanoma of the gallbladder: Does it exist? Report of a case and review of the literature

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Abstract

With the occasion of a case of malignant melanoma of the gallbladder, which appeared to be primary, we have reviewed the literature and the result of this research was that primary melanoma of the gallbladder remains a questionable medical entity. Only few cases of both primary and metastatic gallbladder melanoma have been reported so far, and the only agreement is that surgery is the mainstay treatment. The role of adjuvant chemotherapy, hormone therapy or immunotherapy for both primary and metastatic disease remains undefined.

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Key words: Neoplasms of the gallbladder; Gallbladder; Metastatic melanoma

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INTRODUCTION

Having a biopsy specimen histologically proving melanoma of the gallbladder, it is difficult to determine whether it is a primary or secondary tumor. Even though gallbladder and this metastatic lesion comprises between 50%-66% of gallbladder metastasis^[1], the occurrence of metastasis in the gallbladder is rare and has only been reported in the literature exceptionally^[2-7]. Both primary melanoma in

the gallbladder and isolated metastasis to the gallbladder, are not common events. In fact, primary melanoma in the gallbladder is an extremely rare condition and its existence is still questioned by some. According to the literature, primary malignant melanoma in the gallbladder is a controversial issue, and it is still far from clear whether primary melanoma of this organ is a definite entity in itself. Herein, we present a patient in whom malignant melanoma developed initially in the gallbladder. There was no evidence of another acceptable primary site in postoperative investigation and no previous history could be obtained of a pigment cutaneous lesion, which had been treated or regressed.

CASE REPORT

A 38-year old woman presented with a 36-h history of right upper abdominal pain associated with nausea, vomiting and a temperature 38.6°C. Physical examination revealed the presence of severe tenderness in the right upper quadrant of the abdomen. The gallbladder was not palpable. The hematological tests showed an elevation of her white blood cell count (WBC) to 13800/mm³ with neutrophilia. Liver function studies were normal; these laboratory findings disclosed the following values (the normal reference range is given in parentheses): serum aspartate aminotransferase, 21 IU/L (< 30 IU/L); alanine aminotransferase, 19 IU/L (< 30 IU/L); serum alkaline phosphatase, 184 IU/L (100-320 IU/L); serum g-glutamyl transpeptidase, 30 IU/L (16-73 IU/L); and the serum bilirubin, 0.7 mg/dL (< 1 mg/dL). Ultrasound examination of the gallbladder did not demonstrate lithiasis but a polypoid mass without acoustic shadowing (Figure 1). The patient underwent open cholecystectomy. The specimen was opened and a polypoid mass measuring 1.5 cm × 2.0 cm of dark-yellow color was revealed. The postoperative course of the patient was uneventful, but the histological report indicated a melanoma of the gallbladder (Figures 2 and 3). In H&E stain the diagnosis of a primary melanoma was suspicious, confirmed by immunostaining (HMB-45). After that, a meticulous medical history, physical examination and imaging check did not reveal any other primary focus of the disease. No evidence of the disease was marked after six months in the periodical control, however, four months later the patient died from cerebral hemorrhage due to brain metastasis. Although the patient was informed of her disease, she refused any chemotherapy as well as



Figure 1 Ultrasound of melanoma of gallbladder.

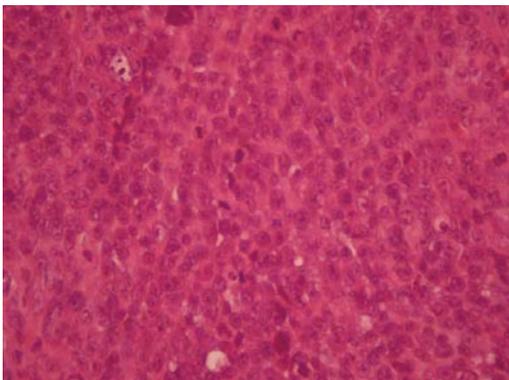


Figure 2 Histological appearance of melanoma of gallbladder stained with HE. $\times 400$.

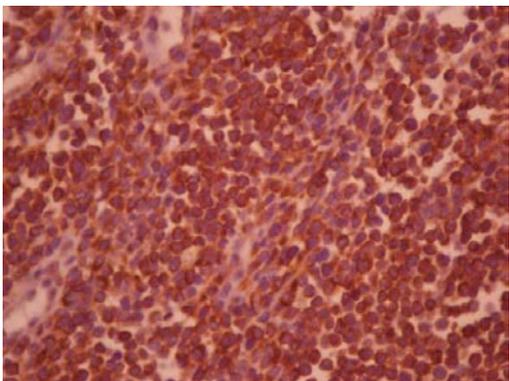


Figure 3 Neoplastic cells immunostained with anti-HMB-45 antibodies. $\times 400$.

the proposed transplenic immunostimulation^[8,9].

DISCUSSION

Theoretically, according to histogenesis, melanoblasts have not yet been demonstrated in organs of endodermal origin^[10] and as gallbladder is an endodermal derivative, it is unlikely that melanoma arises from this organ or other visceral structures. On the other hand, non neoplastic melanoblasts resulting from migration of melanin-producing cells from the neural crest to endodermal derivatives during embryologic development explains the presence of me-

lanocytes within their mucosa^[11-13] and supports the possibility of developing primary melanomas at these sites^[14]. Therefore, primary melanoma of the gallbladder is at least theoretically possible^[4,15]. Even though primary melanoma of the gallbladder remains a debatable clinical entity, reports on this subject continue to accrue. In 1907 the first primary melanoma of the gallbladder was described by *Wieting* and *Handt*^[16] and fifty years later *Walsh* reported the first histologically proven case of primary gallbladder melanoma^[17]. Since that time there have been additional reports of primary melanoma of the gallbladder^[12,15,18-20]. In fact, it is very difficult to differentiate primary from metastatic lesions based on histological features. *Mac Fadden et al*, compared primary and metastatic biliary melanomas and found that the two groups displayed remarkable similarity with regard to pathologic findings^[13]. To help in the differentiation between primary and metastatic melanoma of the gallbladder, according to the literature, the following clinical and pathological criteria should be fulfilled: (1) any other obvious primary site must be excluded by medical history and laboratory investigation, and (2) the tumor, either papillary or polypoid, must be solitary arising from the mucosa of the gallbladder and display junctional activity^[2-4]. Junctional activity, i.e. intraepithelial extension in the mucosa overlying the tumor is considered as significant finding in the diagnosis of the primary melanoma of mucosal surfaces^[11,21]. However, the presence of atypical melanocytes in the adjacent epithelium of invasive tumor does not necessarily define a primary tumor^[22]. Although the presence of “junctional changes” of the mucosa is still controversial, the presence of a solitary polypoid tumor within the lumen of the gallbladder accompanied by junctional activity within the tumor and the absence of an antecedent history of melanoma, or lack of another obvious primary site, are factors that suggest the gallbladder as the primary site of origin. Finally, even today, the establishment of primary histogenesis in these cases is contingent upon a detailed medical history of the patient, a thorough cutaneous and ophthalmic inspection and a radiologic control. However, there always exists the possibility that a regressed primary tumor is a hidden source of gallbladder melanoma. In regard to clinical presentation, involvement of the gallbladder rarely produces symptoms, which could explain the paucity of cases reported in the literature^[23], however, as it is usually reported including our case, acute cholecystitis without the presence of stone in the majority of cases was the initial clinical manifestation and the reason for emergency admission in the hospital. In contrast to gallbladder cancer, melanoma of the gallbladder does not seem to be associated to cholelithiasis. In 1999 *Dong et al*, after having reviewed the literature, found that gallstones appear to be only incidentally and namely in 21.1% of cases (4 of 19 patients) with primary lesions and 27.3% of cases (3 of 11 patients) with symptomatic metastatic disease and this was consistent with the results of their series^[24]. Several diagnostic modalities have been employed to investigate gallbladder lesions. Ultrasound is by far the most useful tool in this attempt. Upper ultrasound abdominal examinations are frequently requested in patients with thick high grade malignant melanoma or clinical suspicion of metastasis. Ultrasound may document metastatic lesions

within the gallbladder. However, polypoid lesions in the lumen of the gallbladder present a focal thickening of the gallbladder wall without acoustic shadowing, due to their lower density in relation with gallstones. As autopsy studies have confirmed the incidence of the gallbladder metastasis from malignant melanoma to be 15%, a careful review of the gallbladder is advocated when abdominal ultrasound examination is performed on patient with malignant melanoma. Ultrasound helps to distinguish between metastasis and benign polyps. The importance of size of the lesion is obvious as 94% of the benign lesions are less than 1 cm in diameter, whereas, 88% of malignant lesion are more than 1 cm^[25]. If the presence of vascular flow within the mass itself is documented, the usefulness of colour-flow Doppler ultrasonography is evident^[26]. Computed tomography is of some value especially in detecting metastatic disease.

Surgical excision is the treatment of choice of both primary and metastatic melanomas of gallbladder. During surgery a thorough search is needed in order to detect any possible abdominal metastasis, as the majority of gallbladder metastases are totally asymptomatic. Although in most cases these metastases are in the form of multiple serosal implants, there are intraluminal metastases involving the mucosa and this fact creates difficulties in detecting these metastases during surgery. Although the mainstay of treatment is surgical removal of the gallbladder, we believe that study of the clear role of adjuvant chemotherapy, hormone therapy or immunotherapy at increasing the survival rate is necessary. Basically, the presence of multiple metastases is an indication of systematic chemotherapy. Till today, the usefulness of adjuvant chemotherapy for both primary and metastatic melanomas remains to be clarified, as only one controlled trial has shown significant improvement in response rate mid survival with the combination dacarbazine and tamoxifen^[27]. Despite appropriate therapy, the diagnosis of either condition portends a poor prognosis with few patients surviving more than two years. Furthermore, according to *Langley*, the prognosis of metastatic melanoma is dismal and the average survival is 7 mo^[28].

In conclusion, firstly, the outlook of patients with gallbladder melanoma remains bleak and secondly, the existence of primary melanoma of the gallbladder is still questionable, and certainly will not be settled by this article.

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CASE REPORT

Multiple small bowel ruptures due to ischemic enteritis: A case report

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Abstract

A rare case of multiple small bowel ruptures due to ischemic enteritis (ISE) is reported. The patient was admitted to the hospital with acute abdominal pain followed by bloody diarrhoeas. Preoperative colonoscopic findings were similar to those presented in Crohn's disease. Intraoperatively, ischemic lesions and multiple ruptures were localized at the jejunum and the proximal ileum. Histopathological examination of the resected bowel segment established the diagnosis of ISE. Although ISE is not common, concurred multiple ruptures of the small bowel is a rare but actual complication.

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Key words: Multiple small bowel ruptures; Ischemic enteritis

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INTRODUCTION

Ischemic enteritis (ISE) is caused either by interruption or significant decrease of the arterial inflow to the small intestine. Most patients are elderly, i.e., over sixty years of age. Younger patients, however, especially those with diabetes, lupus erythematosus or sickle-cell anaemia, may also present with ISE. Definite diagnosis of ISE cannot be established before histopathological results are obtained. ISE seems to be a rapid, progressive disease resulting in a 95% mortality rate^[1]. We report a rare case of acute abdo-

men due to multiple ruptures of the small bowel. ISE was proved to be the cause of the ruptures.

CASE REPORT

A 62-year old male presented with a two-month history of abdominal pain, watery diarrhoeas and occasionally fresh intestinal bleeding. Colonoscopy revealed aphthous ulcers both in the ascending colon and in the cecum. A small bowel series showed "tile-paved" images with multiple ulcers in the ileum. Furthermore, gastroscopy revealed the presence of aphthous ulcers in both the stomach and the duodenum. These ulcers were similar to those found in the colon. Biopsy specimens were taken from the stomach, the ileum, the cecum and the rest of the colon. The diagnosis of Crohn's disease was suspected and anti-Crohn's drug therapy was started. The histopathology results 8 d later, however, excluded Crohn's disease and revealed signs of focal enteritis without inflammatory reaction in the submucosa. Ten days after the first admission, the patient presented again in the emergency department suffering from acute abdominal pain accompanied by diarrhoeas and moderate intestinal bleeding. Physical examination revealed acute abdomen and the plain abdominal X-rays showed free air under the diaphragm. CT scanning confirmed liquid and air accumulation in the abdominal cavity.

The patient underwent emergency laparotomy. Intraoperatively, more than twenty micro-perforations localized in the mesenteric surface of the small bowel were found extending from the end of the jejunum to the middle of the ileum. Intestinal resection (about 60 cm in length) followed by side-to-side anastomosis was performed. Four days later, the patient developed similar symptoms of acute abdomen. On reoperation, numerous micro-perforations in both the jejunum and the remaining ileum were found. An additional small bowel resection (about 80 cm in length) was performed, followed by jejunostomy.

On the third postoperative day the patient was operated on again due to the same symptoms. Multiple perforations were encountered once more in the remaining small bowel and an additional bowel resection (approximately 120 cm in length) was performed. Within three days the patient developed renal and hepatic failure and died 2 d later. Macroscopically, the intestinal lesions were characterized by mucosal ulceration of variable length along the resected segments of small bowel, progressing to full thickness necrosis of the intestinal wall. Histopathological examina-

tion revealed numerous scattered segments of acute necrotizing enteritis with annular strictures and relatively shallow ulcers.

DISCUSSION

Two types of ISE have been described in the literature: occlusive and non-occlusive. Embolism or thrombosis of the superior mesenteric artery are the causes of occlusion in 30% and 25% of the patients respectively. Thrombosis of the superior mesenteric vein is responsible for occlusion in 20% of the patients. Non-occlusive ISE occurs in the remaining patients (25%)^[2]. Although the embolus in superior mesenteric artery embolism is usually installed in the middle colic artery, small intestinal branches are also sometimes occluded. The jejunum is affected in 20% and the ileum in 45%-55% of the patients respectively^[3]. Thrombosis of the superior mesenteric artery or vein is considered to be idiopathic in the majority of patients. However, it can also be related to diseases such as portal hypertension, septicaemia, lack of antithrombin III, lack of protein C or S, as well as chronic contraceptive pills intake^[4].

Non-occlusive ISE is due to low blood flow because of cardiac insufficiency, acute myocardial infarction, arrhythmias, dissecting aneurysm of the aorta, septicaemia and diabetes mellitus as well. Decrease of blood flow up to 80% of its normal value occurs due to prolonged vasoconstriction of the mesenteric vessels^[2]. According to Feurle *et al*^[5], analogous to ischemic colitis, an entity of acute ischemic small bowel enteritis exists, so that mesenteric ischemia apparently can induce a clinical syndrome of "regional enteritis", as occurred in our patient.

Early symptoms (acute abdominal pain and bloody diarrhoeas) and late complications (intestinal necrosis, peritonitis, septicaemia and shock) in our patient, were similar to those presented in the literature^[6].

The accuracy of angiography in localizing the site of embolism or thrombosis of the superior mesenteric artery is more than 95%. Its main advantage is that the catheter may remain in the artery, so that vasodilatation substances can be properly given^[7]. Angiography was not performed in our case due to acute abdominal symptoms. These symptoms led the patient to emergency laparotomy. The accuracy of CT scans in diagnosing mesenteric artery or vein thrombosis is approximately 80%. For embolism the rate is 45%-55%^[8,9]. CT scans, however, fail to establish the diagnosis in the majority of ISE cases, and this happened

in our patient as well. ISE due to superior mesenteric artery embolism can be treated by arteriotomy and embolectomy. The patient should undergo an aorto-mesenteric bypass in the case of thrombosis of the superior mesenteric artery. In initial stages of superior mesenteric vein thrombosis conservative treatment with high doses of heparin is recommended. Unfortunately, in most cases the diagnosis is established during laparotomy, due to acute abdominal symptoms. This occurred in our patient as well^[10].

In conclusion, ischemic enteritis is an uncommon but usually fatal clinical condition. The disease may lead to haemorrhage, intestinal necrosis, multiple small bowel ruptures, peritonitis, septicaemia, shock and death. Immediate diagnosis and proper medical and surgical therapy are crucial for a good prognosis. Unfortunately, a definite diagnosis of the disease is usually established after histopathological results of the resected bowel segment have been obtained. Although the disease is not common, it should be considered in the differential diagnosis of acute abdomen, especially when acute abdominal pain is accompanied by diarrhoea and/or intestinal bleeding.

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CASE REPORT

Splenic arteriovenous fistula and sudden onset of portal hypertension as complications of a ruptured splenic artery aneurysm: Successful treatment with transcatheter arterial embolization. A case study and review of the literature

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Abstract

Splenic arteriovenous fistula (SAVF) accounts for an unusual but well-documented treatable cause of portal hypertension^[1-4]. A case of a 50-year-old multiparous female who developed suddenly portal hypertension due to SAVF formation is presented. The patient suffered from repeated episodes of haematemesis and melaena during the past twelve days and thus was emergently admitted to hospital for management. Clinical and laboratory investigations established the diagnosis of portal hypertension in the absence of liver parenchymal disease. Endoscopy revealed multiple esophageal bleeding varices. Abdominal computed tomography (CT) and transfemoral celiac arteriography documented the presence of a tortuous and aneurysmatic splenic artery and premature filling of an enlarged splenic vein, findings highly suggestive of an SAVF. The aforementioned vascular abnormality was successfully treated with percutaneous transcatheter embolization. Neither recurrence nor other complications were observed.

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Key words: Splenic artery aneurysm; Splenic arteriovenous fistula; Portal hypertension; Transcatheter embolization

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INTRODUCTION

Splenic arteriovenous fistulas (SAVF) represent a rare pathological entity that should be suspected especially in cases of acute portal hypertension not related to chronic liver disease^[1-4]. They may be either asymptomatic or lead rapidly to the development of portal hypertension, with a clinical picture of gastro-intestinal bleeding, diarrhea, ascites or unfavorable heart failure due to hyperdynamic blood flow state^[1-4].

In this report an uncommon case of portal hypertension affecting a multiparous female is discussed. Rupture of a splenic artery aneurysm (SAA) into the splenic vein and formation of a fistulous tract between them consist the underlying pathology. The authors emphasize the positive impact of transcatheter arterial embolization as an effective alternative to surgical intervention in the management of such vascular malformations.

CASE REPORT

A 50-year-old female was referred to our institution for management of an intermittent but intense epigastric pain and a feeling of gastric fullness that were associated with repeated episodes of haematemesis and melaena during the past twelve days. No history of abdominal (accidental or iatrogenic) trauma was reported. On the other hand, it was notable the fact that the patient had delivered successfully four labors between the ages of 26-40 years old.

Upon admission the patient's abdomen was distended and tender. The liver was not palpable and no signs of jaundice were observed. Abdominal auscultation revealed a systolic bruit located at the left flank. Ascites was also depicted. Full blood cell count exhibited markedly decreased values of hematocrit and hemoglobin. Coagulation studies and liver and renal function tests were not affected. Serological markers of hepatitis A, B, C, D were negative and no antibodies were detected. Urgent endoscopy showed multiple bleeding esophageal varices. The stomach was

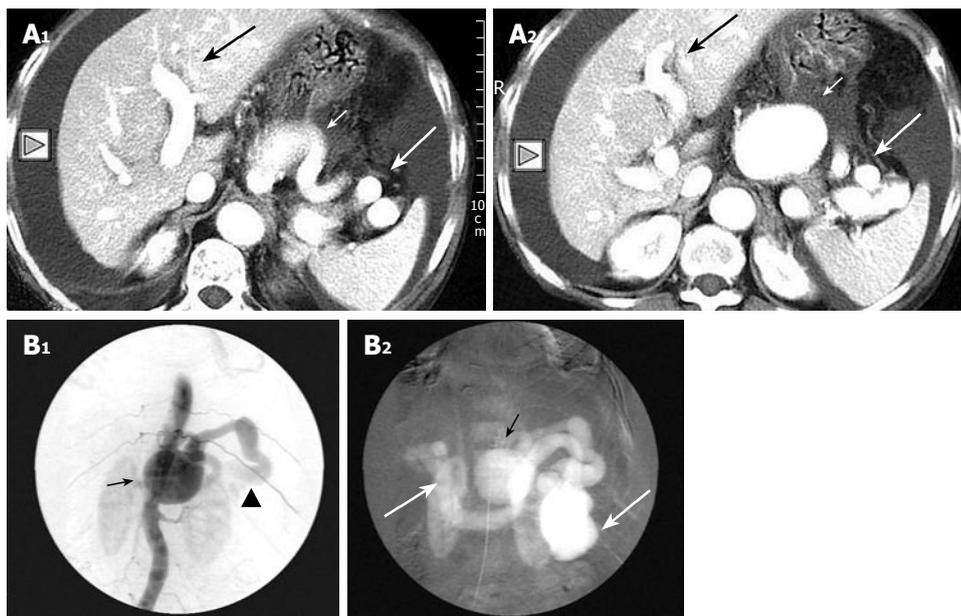


Figure 1 A: Post contrast CT scan reveals a tortuous and aneurysmatic splenic artery of maximum diameter of approximately 52 mm (short white arrow) associated with dilated vessels at the splenic hilum (long white arrow) and early opacification of the portal axis (long black arrow). In addition ascites is present as well (small triangle); B: Celiac angiogram confirms the presence of the splenic artery aneurysm (black arrow) in contiguity with the markedly dilated splenic vein and the premature and intense filling of the splenoportal trunk (black arrowhead and white arrows).

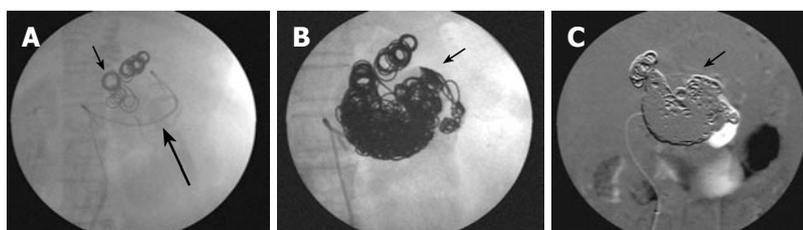


Figure 2 Selective transarterial catheterization (long black arrow) and embolization of the aneurysmal sac with numerous adequate metallic macrocoils (small black arrows) resulted in full occlusion of the sac and the fistulous tract enabling thus the reduction of the pressure in the splenoportal circulation.

found full of blood and the gastric mucosa was red irritated and edematous; findings consistent with mild portal gastritis. The endoscopic variceal ligation that immediately followed only controlled the bleeding temporarily.

Contrast enhanced CT scan of the upper abdomen demonstrated an aneurysmal dilated tortuous splenic artery of maximum diameter of 52 mm, an engorged splenic vein along with simultaneous opacification of the splenic vessels as well during the arterial phase (Figure 1A). Ascites was also confirmed. The aforementioned imaging features strongly supported the case of an SAA that was connected to the splenic vein through a fistula and caused an abrupt elevation of the portal pressure. Celiac and splenic arteriographies were carried out and showed a dilated splenic artery with a large aneurysm combined with premature portal and splenic vein filling and marked opacification of the splenoportal axis (Figure 1B).

The patient's condition remained unstable due to continuing bleeding and transcatheter embolization was scheduled. By using the transfemoral route the aneurysmal sac was accessed and subsequently detachment of adequate metallic macrocoils was performed resulting thus in full occlusion of the sac, cessation of the hyperkinetic portal flow and successful control of the gastro-intestinal bleeding (Figure 2).

The patient had an uncomplicated recovery and was discharged eight days later in stable clinical condition. At the time of this writing, 10 mo later the patient remains free of symptoms with no evidence of post-procedural recurrence or complications.

DISCUSSION

SAA was first described by Beaussier in 1770^[5]. According to autopsy studies its prevalence ranges from 0.01% to 10.4%. SAAs are found incidentally on 0.78% of angiograms and in 7.1% of patients with cirrhotic portal hypertension^[6]. Splenic artery accounts for the third most common abdominal vessel site that is affected by the aneurysmal disease after infra-renal aorta and iliac artery^[7,8].

Splenic artery aneurysm formation relies upon structural incompetence of the connective tissue of the arterial wall to secure the integrity of the vessel lumen^[5,6,8]. Associated risk factors include portal hypertension, connective tissue disorders, congenital abnormalities, trauma and infection^[6]. Unlike other true visceral aneurysms atherosclerosis does not play a leading role in splenic artery aneurysmal dilatation. They are most commonly encountered among females (female to male ratio: 4/1) especially the multipara^[6].

It has been postulated that increased visceral blood flow and gestational hormones during pregnancy alter pathologically the structure of the arterial wall by causing fragmentation of the elastic fibers of the media and loss of smooth muscle^[6,8]. As parity increases these factors have a cumulative effect predisposing thus to aneurysmal formation^[6]. Hence, the fact that this patient had four successful pregnancies can justify the progressive growth of a splenic artery aneurysm.

Splenic artery aneurysm may present as an incidental finding on imaging modalities or with abdominal pain^[6].

It has been also reported a lifetime risk of rupture of 2%-10% with an associated mortality of 25%^[9]. These rates increase remarkably among pregnant women whereas the estimated risk of rupture can reach up to 50% with related maternal and fetal mortality of 70% and 90% respectively^[6]. The rupture may take place either into the gastrointestinal tract and peritoneal cavity or into the splenic vein resulting in splenic arteriovenous fistula development.

Splenic arteriovenous fistulas represent an unusual clinical entity that was first reported by Wiegert in the 19th century^[1-4]. Their majority occurs more commonly after rupture of an SAA into the corresponding vein but their origin can be congenital, traumatic (iatrogenic or accidental) or even infectious^[1-4].

Despite the fact that SAVFs may stay free of symptoms for a long period of time, the hemodynamic changes that develop owing to the arteriovenous shunt may lead to a sudden increase of portal vein pressure. In contrast, chronic liver disease follows a more time-consuming process before ending in portal hypertension^[1].

The clinical profile of splenic arteriovenous fistula mainly consists of upper abdominal pain, gastro-intestinal bleeding and secretory diarrhea^[1-4]. These features are attributed to the abrupt increase of the mesenteric venous pressure^[1,3]. An abdominal bruit, usually of the "machinery" type, is often heard over the left flank^[1,3,4] and although its presence can raise a high index of clinical suspicion, it is encountered only in 30% of cases^[4].

Although abdominal ultrasound combined with the Color Doppler technology can document blood flow abnormalities, splenomegaly and exclude liver parenchymal disease, the established imaging modality in detecting SAVFs is selective celiac or splenic arteriography. The method localizes with accuracy the vascular abnormality and contributes greatly in the assessment of collateral flow pathways^[10]. It is mostly recommended in cases with sudden onset of portal hypertension, abdominal bruit and absence of chronic liver disease^[4]. The patient's medical history should be always taken into account.

On the basis of a well-established diagnosis immediate intervention is rendered mandatory in order to avoid unfavorable complications that will adversely affect prognosis. Splenic artery aneurysms should be promptly treated in high risk patients with symptomatic or expanding aneurysms, women of childbearing age and also patients undergoing liver transplantation^[5,11].

Traditionally surgical and or laparoscopic resection with or without splenectomy is employed in the treatment of SAAs^[5-7,9-12]. However, this patient carried a substantial surgical risk owing to the continuing variceal bleeding and the high possibility of aneurysmal rupture, during surgery, due to its size and the elevated pressure in the splenoportal axis. In addition, surgical approach of SAVFs presents often with technical difficulties and potential hazards of failure because of the distal site of the lesion and the formation of adhesions and numerous portal col-

laterals^[4]. As a result, endovascular techniques emerged as an alternative option. Aneurysmal exclusion using a stent graft was not considered as a feasible solution mostly due to the presence of the fistula and the tortuosity of the splenic artery. In this case successful control of the variceal bleeding and cessation of the hyperkinetic blood flow in the splenoportal axis were achieved by selective catheterization of the sac and detachment of adequate metallic coils that promote permanent thrombosis. Unlike surgical techniques transcatheter arterial embolization is a less invasive, relatively low-risk, rapid procedure that can be easily applied regardless of the location of the vascular malformation^[4,12]. It does not necessitate splenectomy avoiding thus immunologic deficits and a lifelong risk of sepsis^[13]. The aforementioned advantages of transcatheter arterial embolization over surgery support strongly its efficacy in treating safely patients with splenic artery aneurysm complicated with arteriovenous fistula^[4,12,13].

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Treatment of solitary gastric carcinoid tumor by endoscopic polypectomy in a patient with pernicious anemia

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INTRODUCTION

Pernicious anemia is characterized by type A (autoimmune) atrophic gastritis, megaloblastic anemia, and neurological and psychiatric findings. It is the most common cause of cobalamin deficiency due to a loss of acid-secreting parietal cells in the gastric body and fundus^[1]. It is known that patients with pernicious anemia have a higher risk to develop gastrointestinal malignancies such as gastric adenocarcinoma and carcinoid tumors, or oesophageal squamous cell carcinoma^[1-4].

Gastric carcinoid tumors, which arise from hyperplasia of enterochromaffin-like (ECL) cells in the gastric mucosa, comprise 1%-3% of gastric neoplasms^[5-7]. Type I carcinoid tumors are associated with ECL cell hyperplasia, hypergastrinemia, achlorhydria, and chronic atrophic gastritis, with or without pernicious anemia^[8,9]. Type I carcinoid tumors are detected approximately in 1%-7% of the patients with pernicious anemia^[1,3,9,10]. Most of the gastric carcinoid tumors are multifocal, solitary tumors are rare^[3,11-14].

This report describes the treatment of a solitary gastric carcinoid tumor by endoscopic polypectomy in a patient with pernicious anemia.

CASE REPORT

A 48-year-old female patient who was suffering from fatigue for three months was admitted to the hospital. She had no gastrointestinal symptoms, and she negated alcohol abuse. Physical examination revealed pallor, jaundice, and atrophic glossitis. A grade-2 systolic ejection murmur was auscultated on the pulmonary area. Laboratory findings were a hemoglobin level of 59 g/L, a white blood cell count of 2400/mm³, a platelet count of 76 000/mm³, a mean erythrocyte volume of 99 fL, and a reticulocyte count of 2.5%. Peripheral blood smear examination revealed 42% neutrophils, 40% lymphocytes, 12% monocytes, 6% eosinophils, and 3% normoblasts. Moreover, macrocytosis, aniso-poikilocytosis, Howell-Jolly bodies, Cabot ring in erythrocytes, and hypersegmentation in neutrophils were observed. Bone marrow aspiration revealed erythroid hyperplasia (E/M ratio: 6/1), evident megaloblastic changes, and nucleo-

Abstract

Type I gastric carcinoid tumors result from hypergastrinemia in 1%-7% of patients with pernicious anemia. We diagnosed pernicious anemia in a 48-year-old female patient with complaint of fatigue for three months. She had no gastrointestinal symptoms. Endoscopic examination of the upper gastrointestinal tract revealed atrophic gastritis and a polypoid lesion in the corpus of 3-4 mm in size. Endoscopic polypectomy was performed. Histopathological examination of the specimen revealed positive chromogranin A and synaptophysin stainings compatible with the diagnosis of a carcinoid tumor. Serum gastrin level was increased, urinary 5-hydroxyindoleacetic acid was within the normal range. There was no other symptom, sign, or laboratory finding of a carcinoid syndrome in the patient. No metastasis was found with indium-111 octreotide scan, computed tomographies of abdomen and thorax. Type I gastric carcinoid tumors are only rarely solitary and patients with tumors < 1 cm in size may benefit from endoscopic polypectomy.

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Key words: Solitary; Pernicious anemia; Gastric carcinoid tumor; Endoscopic polypectomy

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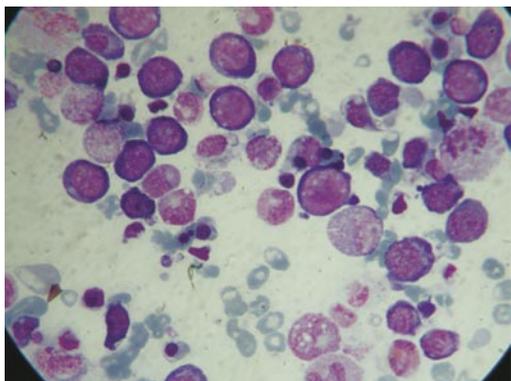


Figure 1 Megaloblastic changes in the bone marrow.

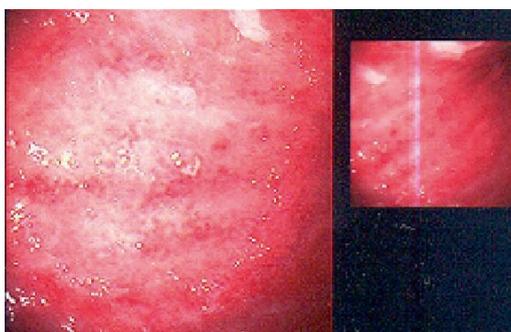


Figure 2 Polypoid appearance and atrophic gastritis at endoscopy.

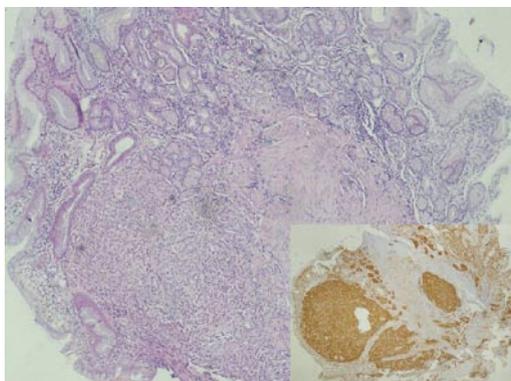


Figure 3 Carcinoid tumor positive stained with hematoxylin-eosin A and synaptophysin (x 40).

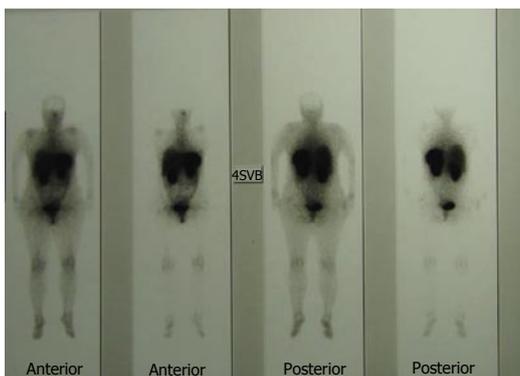


Figure 4 Indium-111 octreotide scan of the patient.

cytoplasmic dissociation in normoblasts (Figure 1). While iron parameters were normal, the levels of vitamin B₁₂ and homocysteine were 62 ng/L (N: 200-900 ng/L) and 91 μmol/L (N: 0-12 μmol/L), respectively. Anti-parietal cell antibody was positive. The biochemical values were normal except those for lactate dehydrogenase 1635 IU/L, total bilirubin 44 mg/L, and indirect bilirubin 34 mg/L. Thyroid function tests were normal, too. By performing upper gastrointestinal tract endoscopy, atrophic gastritis in fundus and corpus and a polypoid lesion in the corpus of 3-4 mm in size were seen (Figure 2). *H. pylori* were not detected with rapid urease test. Endoscopic polypectomy was performed. By histopathological examination, a carcinoid tumor was diagnosed, mainly on the basis of positive stainings for chromogranin A and synaptophysin. *H. pylori* was negative (Figure 3). The levels of fasting serum gastrin and 24 h urinary 5-hydroxyindoleacetic acid were 2000 ng/L and 12.6 μmol (N: 10.4-31.2 μmol), respectively. Spiral computed tomographies of thorax and abdomen were normal. Indium-111 octreotide scan revealed a normal distribution of tracer activity (Figure 4). Electrocardiography, transthoracic Doppler echocardiography, chest X-ray, and gastric endoscopic ultrasonography were normal. Endoscopic re-examination of the stomach was performed and multiple biopsies were obtained from the polypectomy site. The second pathological examination failed to detect any remnant of the carcinoid tumor or neuroendocrine cell hyperplasia. Treatment with vitamin B₁₂ at a dose of 1000 μg/d orally was started^[15]. After 6 mo follow-up, the patient was well, and her whole blood counts and the vitamin B₁₂ level were normal.

DISCUSSION

Type I gastric carcinoid tumors result from hypergastrinemia in patients with pernicious anemia. Hypergastrinemia is the result of achlorhydria and atrophic gastritis. Hypergastrinemia acts as a trophic factor for ECL cells, resulting in hyperplasia in gastric mucosa^[6,9-11,16,17]. Serum gastrin levels usually range from 740-4000 ng/L in those patients^[17]. Although most of those patients are asymptomatic, dyspepsia, pain, nausea, unexplained weight loss, gastrointestinal bleeding, or anemia may be seen^[11,13,17]. These carcinoid tumors are usually localized in the gastric fundus or corpus and are less than 2 cm in diameter. Often, they are multifocal. Nodal and hepatic metastasis occur in 2% of the patients^[3,11,12,14,17]. Solitary gastric carcinoid tumors that like our patient were reported infrequently^[11,13,14,17]. Type II gastric carcinoid tumors, a very rare type, occurs in patients with Zollinger-Ellison syndrome. Most of carcinoid tumors are associated with multiple endocrine neoplasia (MEN) type I. Hypergastrinemia and ECL cell hyperplasia are seen in type II gastric carcinoid tumors. They are multifocal, and their sizes are between 1-2 cm. Local lymph node metastasis can produce^[5,6,11]. Type III gastric carcinoid tumors are sporadic, they are not associated with hypergastrinemia, and, in contrast to types I and II, they are invasive large solitary tumors. Carcinoid tumors larger than 3 cm cause metastasis in 66%

of the patients while metastasis is detected in only 10% of patients with single tumors smaller than 1 cm^[11,17,18].

In our patient, pernicious anemia was diagnosed because of macrocytic anemia, megaloblastic changes in the bone marrow, a decreased vitamin B₁₂ level, an increased homocysteine level, and atrophic gastritis. The pathological examination of the polyp revealed a solitary gastric carcinoid tumor. Serum gastrin level was high, and urinary 5-hydroxyindoleacetic acid level was normal. There were not symptoms, signs, or laboratory findings of a carcinoid syndrome in our patient. Because of hypergastrinemia in the absence of a Zollinger-Ellison syndrome or MEN type I, the final diagnosis was type I gastric carcinoid tumor.

The best approach modality to the patients with type I gastric carcinoid tumors is endoscopic resection especially in tumors smaller than 1 cm^[17,19]. Antrectomy/total gastrectomy is recommended in cases with multiple tumors larger than 1 cm, and with abdominal symptoms such as unexplained weight loss, or aggravation of anemia^[6,17,19,20].

We performed endoscopic polypectomy and re-evaluated the patient endoscopically with repeated biopsies. Metastasis was not detected in computed tomographies, pathological examinations, and octreotide scan. Thus, antrectomy/total gastrectomy was not performed. Annual follow-up with endoscopy and computed tomography are planned for the patient. In pernicious anemia, the incidence of gastric carcinoid tumors is increasing as a result of the careful endoscopic and pathological examinations. Type I solitary gastric carcinoid tumors are infrequently detected solitary and endoscopic polypectomy may cure the patient if the tumor is smaller than 1 cm. A follow-up program should be scheduled for patients who underwent polypectomy for any metastasis.

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CASE REPORT

Computed tomographic findings of trichuriasis

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Abstract

In this report, we present computed tomographic findings of colonic trichuriasis. The patient was a 75-year-old man who complained of abdominal pain, and weight loss. Diagnosis was achieved by colonoscopic biopsy. Abdominal computed tomography showed irregular and nodular thickening of the wall of the cecum and ascending colon. Although these findings are nonspecific, they may be one of the findings of trichuriasis. These findings, confirmed by pathologic analysis of the biopsied tissue and Kato-Katz parasitological stool flotation technique, revealed adult *Trichuris*. To our knowledge, this is the first report of colonic trichuriasis indicated by computed tomography.

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Key words: *Trichuris trichiura*; Colitis; Large bowel; Imaging findings; Computed tomography

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INTRODUCTION

Trichuris trichiura (*T. trichiura*), a whipworm, is the third most common nematode worldwide after *Ascaris* and *Enterobius*^[1]. First described by Roederer in 1761, the whipworm is found primarily in tropical climates characterized by poor sanitation^[1]. Whipworm infection usually causes no clinical symptoms, although a severe infection can cause abdominal pain, diarrhea, constipation, weight loss, and anemia^[2-5].

In the patient described in this report, we identified irregular and nodular marked thickening of the wall of the cecum and ascending colon as a CT finding of trichuriasis. To our knowledge, this is the first report of colonic trichuriasis revealed by that modality. A follow-up CT scan showed that the colonic manifestations of trichuriasis had resolved six months after the conclusion of the medical treatment.

CASE REPORT

A 75-year-old man with suspected colon carcinoma was referred to our radiology unit for evaluation by computed tomography (CT). He complained of abdominal pain, constipation, and a weight loss of 5 kg in the prior two months. Physical examination revealed mild tenderness in the right lower abdomen. The patient's white blood cell count was $13.9 \times 10^9/L$ (reference intervals, $5-11 \times 10^9/L$) without eosinophilia. The results of other laboratory tests (including biochemical analysis) were within the normal range with the exception of moderate anemia (hemoglobin level, 90 g/L; reference intervals, 140-180 g/L).

CT scans were obtained using a four-channel MDCT scanner (Sensation 4; Siemens, Erlangen, Germany). A mixture of 1000 mL water and 40 mL contrast material was used for bowel opacification. Portal phase CT scans (2.5 mm collimation, 12.5 mm/0.5 s table speed) were acquired at 60 s after contrast material injection.

CT scan of the abdomen showed irregular and nodular marked thickening of the wall of the cecum and ascending colon (Figure 1A, B). Endoscopic examination revealed multiple ulcerated mucosal lesions in the ascending colon. The cecum could not be properly examined because of intestinal content. The results of pathologic analysis of the biopsied tissue, which was obtained colonoscopically from the ascending colon, revealed adult *Trichuris* and the signs of active colitis (Figure 2); these findings were confirmed by Kato-Katz parasitological stool flotation technique^[6]. The patient was subsequently treated with mebendazole 100 mg twice daily for 3 d. On follow-up at the end of the treatment clinical findings had resolved, and a repeat CT scan obtained 6 mo after the treatment revealed a colonic wall thickness within the normal range (Figure 3) and no *T. trichiura* eggs were seen in any stool specimens.

DISCUSSION

Trichuriasis is an intestinal infection found in human beings, which is caused by *T. trichiura*, more commonly known as whipworm because of its whip-like appearance.

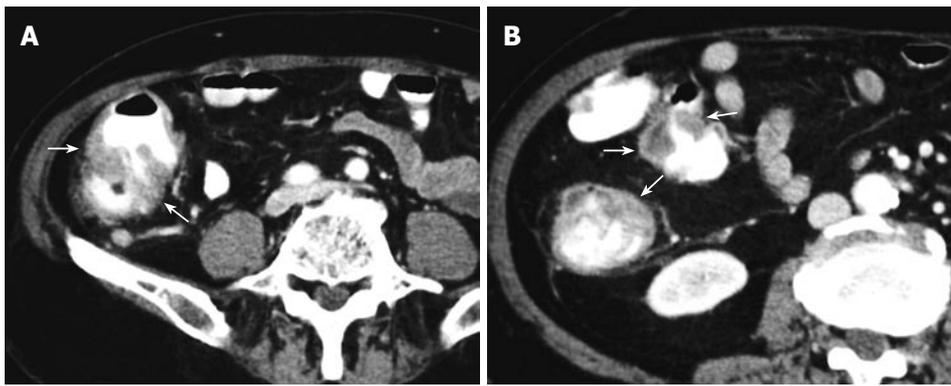


Figure 1 Computed tomographic scans (A, B) of the abdomen. Note the irregular and nodular thickened wall of the cecum and the ascending colon (arrows).

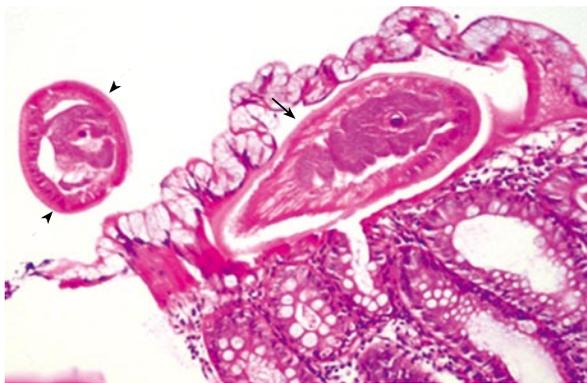


Figure 2 Pathologic examination reveals transverse section of anterior portion of *Trichuris trichiura* (arrow) embedded in superficial colonic mucosa and free body part of *Trichuris trichiura* (arrowheads) extending into the lumen of the colon.

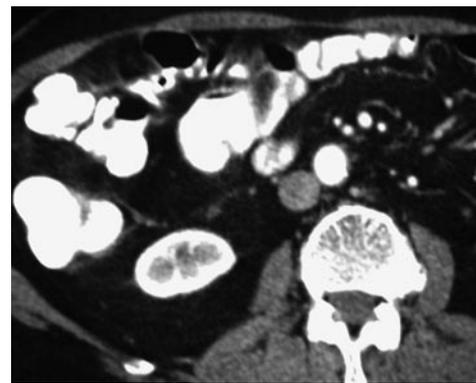


Figure 3 Computed tomographic scan obtained 6 mo after the treatment show a normal colonic wall thickness.

It is characterized by the invasion of the colonic mucosa by the adult *Trichuris* and produces minor inflammatory changes at the sites of localization. Trichuriasis remains a health problem in certain geographic areas. Infection is acquired by the ingestion of embryonated eggs from contaminated drinking water and food. The eggs hatch in the small intestine, and the larvae then enter the intestinal crypts^[1]. After a brief period of maturation, the larvae may migrate to the proximal colon, where they reside and over 1 to 3 mo mature into adults^[1,2,6,7]. The adult *T. trichiura* is a threadlike worm 30 to 50 mm in length. *T. trichiura* penetrates the intestinal mucosa and frequently triggers minor inflammatory changes in the cecum, appendix, and terminal ileum^[7]. Diagnosis is usually based on the identification of typical *T. trichiura* eggs in stool specimens^[6]. Adult whipworm is rarely seen during colonoscopy and colonoscopy can directly diagnose trichuriasis, confirming the threadlike form of worms with an attenuated end^[7-11]. Literature research revealed that imaging findings sometimes similar with a tumor of the colon because of tumor-like appearance of the granulomatous tissue reaction due to *T. trichiura*^[12].

A few reports have described the radiographic appearance of *T. trichiura* on double-contrast barium enema^[13]. Characteristic findings revealed by that modality have included multiple tiny target-shaped or pinwheel-shaped collections of barium that are associated with peculiar s-shaped filling defects thought to be consistent with the shape of the adult male *T. trichiura*^[13]. Colonic

wall thickening is a common and nonspecific finding that occurs in many other conditions such as infections, inflammatory bowel disease and carcinoma. However, knowledge of this entity is helpful for differential diagnosis in the endemic areas of trichuriasis.

We concluded that colonoscopy with biopsy is the gold standard for such disease like trichuriasis. Irregular and nodular thickening of the cecum and the ascending colon on CT scan may be one of the findings of trichuriasis. Although that finding is nonspecific, parasitic infections including trichuriasis should be considered as differential diagnoses, especially in areas in which such infection is endemic.

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Delayed hemorrhage from hepatic artery after ultrasound-guided percutaneous liver biopsy: A case report

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Abstract

Percutaneous liver biopsy is considered one of the most important diagnostic tools to evaluate diffuse liver diseases. Pseudoaneurysm of hepatic artery is an unusual complication after ultrasound-guided percutaneous liver biopsy. Delayed hemorrhage occurs much less frequently. We report a case of pseudoaneurysm of the hepatic artery of a 46-year-old man who was admitted for abdominal pain after 4 d of liver biopsy. The bleeding was controlled initially by angiographic embolization. However, recurrent bleeding could not be controlled by repeat angiography, and the patient died 4 d after admission from multiorgan failure. The admittedly rare possibility of delayed hemorrhage should be considered whenever a liver biopsy is performed.

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Key words: Hemorrhage; Hepatic artery; Percutaneous liver biopsy

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INTRODUCTION

Percutaneous liver biopsy is considered one of the most important diagnostic tools to evaluate diffuse liver diseases. The most common complication of percutaneous liver biopsy is bleeding. The major complication and mortality rates are about 2%-4% and 0.01%-0.33% respectively^[1]. Ultrasonography-guided percutaneous liver biopsy has been shown to increase the diagnostic yield and significantly decrease complications even on outpatients^[2-4].

The less common complication of delayed bleeding after percutaneous liver biopsy, has a higher mortality associated with late recognition^[5]. Pseudoaneurysms of the hepatic artery initially should be managed with angiographic embolization, reserving surgical intervention for failure of embolization^[6]. Here we report a case of fatal delayed hemorrhage from pseudoaneurysm of the hepatic artery after percutaneous liver biopsy. The diagnosis was confirmed by ultrasound and angiography.

CASE REPORT

A 46-year-old man with genotype 1b chronic hepatitis C virus (HCV) infection was evaluated for progressive fatigue and elevated aminotransferases. The hemoglobin, prothrombin time, bleeding time, and platelet count were normal. Abdominal ultrasound was normal and ultrasonography showed no focal hepatic lesion or evidence of cirrhosis. He received a liver biopsy to evaluate the pathologic change before interferon therapy. A percutaneous liver biopsy was conducted under ultrasonographic guidance with a 2.8-mm Menghini-type aspiration needle with one pass. Chronic hepatitis was diagnosed based on histopathological assessment according to a scoring system that includes semi-quantitative assessment of liver disease grading and staging^[7]. Laboratory data and liver biopsy revealed moderate activity (grade 2/4, stage 2/4). After liver biopsy, transient hypotension was noted during the first two hours of in-hospital observation and the patient was discharged 6 h later.

Four days later, the patient complained of right upper quadrant pain radiating to the right shoulder. The abdomen was soft with normal bowel sounds and mild right upper quadrant tenderness without rebound tenderness. The patient denied abdominal trauma. Laboratory results including white blood cell count (WBC): $5.5 \times 10^9/L$; platelet count (PCT): $204 \times 10^9/L$; hematocrit (HCT): 47%; prothrombin time international normalized ratio (INR): 1.0; aspartate aminotransferase (AST): 107 IU/L; alanine aminotransferase (ALT): 145 IU/L; total bilirubin (TBIL): 16 $\mu\text{mol/L}$; alkaline phosphatase (ALP): 128 IU/L; BUN and serum creatinine (CRE): were normal. His blood pressure (BP) was 19.3/11.3 kPa, and heart rate (HR) 69 beats/min. The diagnosis of pseudoaneurysm was established 4 d after the percutaneous liver biopsy by abdominal ultrasonography. The patient suddenly became hemodynamically unstable

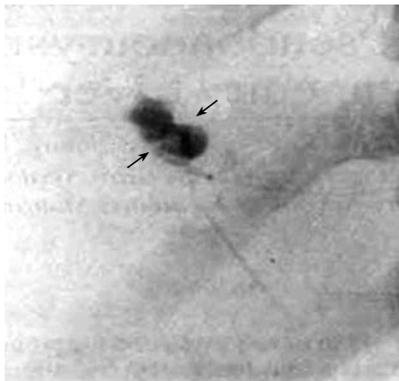


Figure 1 Angiography of the right hepatic artery. Pseudoaneurysm (arrows) in the right hepatic artery branch.

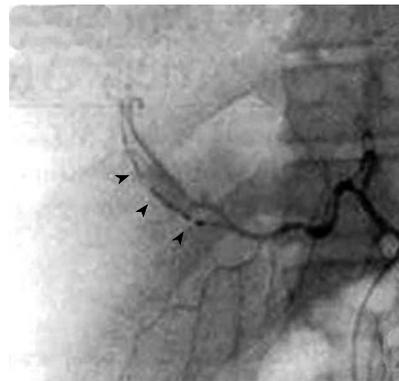


Figure 2 Platinum coils (arrows) are seen filling the feeding branch. Angiography obtained after embolization of the branch leading to and from the pseudoaneurysm.

with an HR 135 beats/min and BP 10.7/5.3 kPa 13 h after admission, which was over 5 d after the percutaneous liver biopsy. The repeat HCT was 39%. The patient was resuscitated and underwent immediate angiography, which showed a pseudoaneurysm in the right hepatic artery branch (Figure 1). The pseudoaneurysm measured approximately 1.8 cm × 2.1 cm. The patient was treated with embolization performed by multiple straight of the right hepatic artery branch (Figure 2). The angiography was repeated 28 h after admission and revealed continued extravasation of blood from the right lateral subcapsular location. These demonstrated new areas of active arterial extravasation with possible complex laceration of the right hepatic lobe. The laboratory tests obtained 33 h after admission showed INR 2.1; ALT: 3970 IU/L; AST: 4910 IU/L; ALP: 290 IU/L; TBIL: 32 μmol/L; albumin (ALB): 17 g/L, CRE: 230 μmol/L; and BUN: 19 mol/L. Soon afterward, the patient received surgery, which revealed right hepatic lobe laceration with hemorrhage. After surgery, the patient's condition continued to deteriorate with a decreasing HCT. Repeated ultrasound of the liver revealed the interval development of multiple cystic lesions in the right hepatic lobe compatible with intrahepatic hematomas. No further surgical intervention was performed because of the diffuse nature of the bleeding. The patient died 4 d after admission from multiorgan failure.

DISCUSSION

Outpatient percutaneous liver biopsy is a common practice in the differential diagnosis and treatment of chronic liver disease. The major complication and mortality rates were about 2%-4% and 0.01%-0.33% respectively^[1]. According to the recent National Institutes of Health (NIH) Consensus Conference on the management of chronic hepatitis C, liver biopsy is recommended before therapy to assess the severity and activity of the liver disease^[18]. Treatment with interferon is recommended if the liver biopsy shows evidence of septal fibrosis and/or moderate-to-severe necroinflammatory changes. More recent view on the issue of performing a liver biopsy in hepatitis C patients advises to perform a liver biopsy in genotype 1 patients, but usefulness of histologic assessment in genotypes 2 or 3 is debated, since these patients have a very good virological response^[9]. At the best, liver biopsy is supported by a grade III recommendation^[9]. Early bleeding

within hours can result from intrahepatic hematoma or laceration of the liver capsule^[1]. Previous studies showed an overall complication rate of 0.28% and fatality rate of 0.03% among 189 085 liver biopsies^[10]. Other reports showed, there were 3 deaths (0.004%) in 68 276 and 19 deaths (0.052%) in 36 786 liver biopsies^[11]. Factors associated with an increased risk of hemorrhage included increasing age, number of biopsy passes and presence of malignancy in the liver^[1,12]. The risk of hemorrhage or complication^[13] was not related to the biopsy needle type or diameter of the needle^[14].

Pseudoaneurysms more commonly occur in patients who are post liver transplant or post cholecystectomy^[15,16]; only 11 pseudoaneurysms occur among 1211 patients undergoing multiple liver biopsies after liver transplantation, whereas only 2 (0.17%) cases were attributed to percutaneous liver biopsy^[17]. This case report demonstrated an unusual complication of hepatic artery pseudoaneurysm from percutaneous liver biopsy. The use of teep mattress sutures for initial control of bleeding is preferred^[18]. After percutaneous liver biopsy with liver diseases, most patients experiencing these events were asymptomatic, and angiographic defects were found in 61% of angiography performed within 1 wk but in 11% of angiography done after more than 1 wk^[16]. The present case showed that clinical features of pseudoaneurysms may range from asymptomatic to hemobilia to massive delayed fatal hemorrhage. Ultrasound with Doppler can be used to diagnose pseudoaneurysm and to evaluate portal blood flow. A celiac or selective hepatic angiography will demonstrate the actual location and size of the pseudoaneurysm^[16,19]. A few previous studies showed that embolization of a hepatic artery pseudoaneurysm had a success rate of 97%, and the occlusion of the more proximal hepatic artery caused by dissection or thrombosis may necessitate a direct percutaneous approach^[15]. Both transarterial and direct percutaneous accesses to pseudoaneurysms have also been successfully employed^[15]. However, direct embolization of the pseudoaneurysm, allowing patency of the hepatic artery, has also been reported^[15]. In this case, bleeding from pseudoaneurysm of the hepatic artery was controlled initially with embolization, but laboratory tests performed after embolization suggest that the patient apparently had ischemic hepatitis and continued to deteriorate from rebleeding. The patient died 4 d after admission from

multiorgan failure. Ischemic hepatitis or shock liver is defined as an extensive hepatocellular necrosis associated with a decrease in hepatic perfusion due to systemic hypotension. Serum aminotransferase levels (ALT and AST) increased rapidly after the ischemic episode and peaked within 1 to 3 d to at least 20 times the upper normal limit. After recovery, aminotransferases returned to near normal levels in 7-10 d of the initial insult^[20].

In conclusion, delayed fatal hemorrhage from pseudoaneurysm of hepatic artery is an unusual complication after percutaneous liver biopsy. Pseudoaneurysms should initially be managed with angiographic embolization^[6], and surgical intervention may be needed if embolization fails.

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Xin-Xin Zhang, Professor

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Meetings

MAJOR MEETINGS COMING UP

First Biennial Congress of the Asian-Pacific Hepato-Pancreato-Biliary Association March, 2007
Fukuoka, Japan
<http://www.congre.co.jp/1st-aphba>

American College of Gastroenterology Annual Scientific
20-25 October 2006
Las Vegas, NV

14th United European Gastroenterology Week, UEGW
21-25 October 2006
Berlin, Germany

APDW 2006: Asian Pacific Digestive Week 2006
26-29 November 2006
Lahug Cebu City, Philippines

EVENTS AND MEETINGS IN THE UPCOMING 6 MONTHS

Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
24-25 March 2006
Sydney - NSW
Falk Foundation e.V.
symposia@falkfoundation.de

10th International Congress of Obesity
3-8 September 2006
Sydney
Event Planners Australia
enquiries@ico2006.com
www.ico2006.com

Easl 2006 - the 41st annual
26-30 April 2006
Vienna, Austria
Kenes International

Prague hepatology 2006
14-16 September 2006
Prague
Foundation of the Czech Society of Hepatology
veronika.revicka@congressprague.cz
www.czech-hepatology.cz/phm2006

12th International Symposium on Viral Hepatitis and Liver Disease
1-5 July 2006
Paris
MCI France
isvhld2006@mci-group.com
www.isvhld2006.com

Falk Symposium 152: Intestinal Disease Part I, Endoscopy 2006 - Update and Live Demonstration
4-5 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

Falk Symposium 153: Intestinal Disease Part II, Immunoregulation in Inflammatory Bowel Disease - Current Understanding and Innovation
6-7 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

ILTS 12th Annual International Congress
3-6 May 2006
Milan
ILTS
www.its.org

Internal Medicine: Gastroenterology
22 July 2006-1 August 2006
Amsterdam
Continuing Education Inc
jbarnhart@continuingeducation.net
6th Annual Gastroenterology And

Hepatology
15-18 March 2006
Rio Grande
Office of Continuing Medical Education
cmenet@jhmi.edu
www.hopkinscme.net

World Congress on Gastrointestinal Cancer
28 June 2006-1 July 2006
Barcelona, Spain
c.chase@imedex.com

International Conference on Surgical Infections, ICSI2006
6-8 September 2006
Stockholm
European Society of Clinical Microbiology and Infectious Diseases
icsi2006@stocon.se
www.icsi2006.se/9/23312.asp

7th World Congress of the International Hepato-Pancreato-Biliary Association
3-7 September 2006
Edinburgh
Edinburgh Convention Bureau
convention@edinburgh.org
www.edinburgh.org/conference

Society of American Gastrointestinal Endoscopic Surgeons
26-29 April 2006
Dallas - TX
www.sages.org

Digestive Disease Week 2006
20-25 May 2006
Los Angeles
www.ddw.org

Annual Postgraduate Course
25-26 May 2006
Los Angeles, CA
American Society of Gastrointestinal Endoscopy
www.asge.org/education

American Society of Colon and Rectal Surgeons
3-7 June 2006
Seattle - Washington
www.fascrs.org

EVENTS AND MEETINGS IN 2006

10th World Congress of the International Society for Diseases of the Esophagus
22-25 February 2006
Adelaide
isde@sapmea.asn.au
www.isde.net

Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
24-25 March 2006
Sydney - NSW
Falk Foundation e.V.
symposia@falkfoundation.de

10th International Congress of Obesity
3-8 September 2006
Sydney
Event Planners Australia
enquiries@ico2006.com
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Easl 2006 - the 41st annual
26-30 April 2006
Vienna, Austria
Kenes International

VII Brazilian Digestive Disease Week
19-23 November 2006
www.gastro2006.com.br

International Gastrointestinal Fellows Initiative
22-24 February 2006
Banff, Alberta
Canadian Association of Gastroenterology
cagoffice@cag-acg.org
www.cag-acg.org

Canadian Digestive Disease Week
24-27 February 2006
Banff, Alberta
Digestive Disease Week Administration
cagoffice@cag-acg.org
www.cag-acg.org

Prague Hepatology 2006
14-16 September 2006
Prague
Foundation of the Czech Society of Hepatology
veronika.revicka@congressprague.cz
www.czech-hepatology.cz/phm2006

12th International Symposium on Viral Hepatitis and Liver Disease
1-5 July 2006
Paris
MCI France
isvhld2006@mci-group.com
www.isvhld2006.com/

Falk Seminar: XI Gastroenterology Seminar Week
4-8 February 2006
Titisee
Falk Foundation e.V.
symposia@falkfoundation.de

European Multidisciplinary Colorectal Cancer Congress 2006
12-14 February 2006
Berlin
Congresscare
info@congresscare.com
www.colorectal2006.org

Falk Symposium 152: Intestinal Disease Part I, Endoscopy 2006 - Update and Live Demonstration
4-5 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

Falk Symposium 153: Intestinal Disease Part II, Immunoregulation in Inflammatory Bowel Disease - Current Understanding and Innovation
6-7 May 2006
Berlin
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14th United European Gastroenterology Week
21-25 October 2006
Berlin
United European Gastroenterology Federation
www.uegw2006.de

World Congress on Controversies in Obesity, Diabetes and Hypertension
25-28 October 2006
Berlin
comtec international
codhy@codhy.com
www.codhy.com

Asia Pacific Obesity Conclave
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New Delhi
info@apoc06.com
www.apoc06.com/

ILTS 12th Annual International Congress
3-6 May 2006
Milan
ILTS
www.its.org

XXX Panamerican Congress of Gastroenterology
11-16 November 2006
Cancun
www.panamericano2006.org.mx

Internal Medicine: Gastroenterology
22 July 2006-1 August 2006
Amsterdam
Continuing Education Inc
jbarnhart@continuingeducation.net

6th Annual Gastroenterology And Hepatology
15-18 March 2006
Rio Grande
Office of Continuing Medical Education
cmenet@jhmi.edu
www.hopkinscme.net

Hepatitis 2006
25 February 2006-5 March 2006
Dakar
hepatitis2006@mangosee.com
mangosee.com/mangosteen/hepatitis2006/hepatitis2006.htm

World Congress on Gastrointestinal Cancer
28 June 2006-1 July 2006
Barcelona, Spain
c.chase@imedex.com

International Conference on Surgical Infections, ICSI2006
6-8 September 2006
Stockholm
European Society of Clinical Microbiology and Infectious Diseases
icsi2006@stocon.se
www.icsi2006.se/9/23312.asp

5th International Congress of The African Middle East Association of Gastroenterology
24-26 February 2006
Sharjah
InfoMed Events
infoevent@infomedweb.com
www.infomedweb.com

7th World Congress of the International Hepato-Pancreato-Biliary Association
3-7 September 2006
Edinburgh
Edinburgh Convention Bureau
convention@edinburgh.org
www.edinburgh.org/conference

13th International Symposium on Pancreatic & Biliary Endoscopy
20-23 January 2006
Los Angeles - CA
laner@cschs.org

2006 Gastrointestinal Cancers Symposium
26-28 January 2006
San Francisco - CA
Gastrointestinal Cancers Symposium Registration Center
gregistration@jpsargo.com

Society of American Gastrointestinal Endoscopic Surgeons
26-29 April 2006
Dallas - TX
www.sages.org

Digestive Disease Week 2006
20-25 May 2006
Los Angeles
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Annual Postgraduate Course
25-26 May 2006
Los Angeles, CA
American Society of Gastrointestinal Endoscopy
www.asge.org/education

American Society of Colon and Rectal Surgeons
3-7 June 2006
Seattle - Washington
www.fascrs.org

71st ACG Annual Scientific and Postgraduate Course
20-25 October 2006
Venetian Hotel, Las Vegas, Nevada
The American College of Gastroenterology

AASLD 57th Annual - The Liver Meeting™
27-31 October 2006
Boston, MA
AASLD

New York Society for Gastrointestinal Endoscopy
13-16 December 2006
New York
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EVENTS AND MEETINGS IN 2007

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20-23 June 2007
Barcelona
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Acknowledgments

Brief acknowledgments of persons who have made genuine contributions to the manuscripts and who endorse the data and conclusions are included.

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Format

Journals

English journal article (list all authors and include the PMID where applicable)

- 1 **Grover VP**, Dresner MA, Forton DM, Counsell S, Larkman DJ, Patel N, Thomas HC, Taylor-Robinson SD. Current and future applications of magnetic resonance imaging and spectroscopy of the brain in hepatic encephalopathy. *World J Gastroenterol* 2006; **12**: 2969-2978 [PMID: 16718775]

Chinese journal article (list all authors and include the PMID where applicable)

- 2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci U S A* 2006; In press

Organization as author

- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462]

Both personal authors and an organization as author

- 5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764]

No author given

- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303]

Volume with supplement

- 7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325]

Issue with no volume

- 8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; **(401)**: 230-238 [PMID: 12151900]

No volume or issue

- 9 Outreach: bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

- 12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wiczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

- 13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ Cell Tumour Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

- 14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

Morse SS. Factors in the emergence of infectious diseases. *Emerg Infect Dis* serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/EID/eid.htm>

Patent (list all authors)

- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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Present as mean \pm SD or mean \pm SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as ν (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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Use SI units. For example: body mass, *m* (B) = 78 kg; blood pressure, *p*(B) = 16.2/12.3 kPa; incubation time, *t* (incubation) = 96 h, blood glucose concentration, *c* (glucose) 6.4 \pm 2.1 mmol/L; blood CEA mass concentration, *p* (CEA) = 8.6 24.5 μ g/L; CO₂ volume fraction, 50 mL/L CO₂ not 5% CO₂; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, etc. Arabic numerals such as 23, 243, 641 should be read 23 243 641.

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Italics

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Heme in intestinal epithelial cell turnover, differentiation, detoxification, inflammation, carcinogenesis, absorption and motility

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Abstract

The gastrointestinal tract is lined by a simple epithelium that undergoes constant renewal involving cell division, differentiation and cell death. In addition, the epithelial lining separates the hostile processes of digestion and absorption that occur in the intestinal lumen from the aseptic environment of the internal milieu by defensive mechanisms that protect the epithelium from being breached. Central to these defensive processes is the synthesis of heme and its catabolism by heme oxygenase (HO). Dietary heme is also an important source of iron for the body which is taken up intact by the enterocyte. This review describes the recent literature on the diverse properties of heme/HO in the intestine tract. The roles of heme/HO in the regulation of the cell cycle/apoptosis, detoxification of xenobiotics, oxidative stress, inflammation, development of colon cancer, heme-iron absorption and intestinal motility are specifically examined.

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Key words: Absorption; Heme; Uptake; Release; Heme oxygenase; Oxidant; Cytoprotection; Inflammation; Cancer; Detoxification

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INTRODUCTION

The lumen of the intestine mucosa is predominately

covered with epithelial cells called enterocytes which are responsible for the terminal digestion and absorption of nutrients. These cells have a limited lifespan before being replaced by cells derived from the crypt region^[1]. There is also evidence of apoptosis within the crypt, presumably in response to excess cellular proliferation, cytotoxicity or genomic damage^[2]. Surviving cells undergo apical migration, limited cell replication, commitment and differentiation^[1]. The process of differentiation is gradual, characterised by the accumulation of cell-specific products in the upper crypt region and attaining the mature phenotype in the lower to middle-villus region. Recent evidence indicates that heme is important in intestinal development as well as maintaining the mucosal barrier and protecting the body from invasion and the damaging consequences of ingested xenobiotics. However, heme in the colon may irritate the mucosa and derange the normal rates of proliferation/exfoliation, circumstances that raise the probability of colon cancer. Heme is also an important source of body iron and how it is absorbed by the enterocyte is considered in this article, as well as the role heme plays in intestinal motility. It needs to be recognised that an in depth focus on each of these components is outside the scope of this review, rather it is our intention to provide the general reader with evidence and interpretations supporting the markedly varied involvement of heme in intestinal function.

HEME BIOSYNTHESIS AND HEME OXYGENASE (HO) (EC 1.14.99.3)

Heme biosynthesis

Heme biosynthesis involves 8 enzymes, four localised to the cytoplasm and the others in the mitochondrial matrix^[3-5] and is regulated by the first enzyme in its synthesis aminolevulinic acid synthase^[6] (Figure 1A). Heme biosynthesis also requires iron, which in the intestinal crypt is derived from the plasma by the activity of the transferrin receptor operating in collaboration with the hemochromatosis protein (HFE)^[7] (Figure 1B). Although heme synthesis is highest in the crypt epithelium it continues along the length of the crypt-villus axis. As the cells leave the crypt region iron appears to be acquired from the diet since dietary iron deficiency reduces the heme content of villus enterocytes, and in villus cells transferrin receptor has 25% the activity of crypt epithelium^[8,9] (Figure 1B).

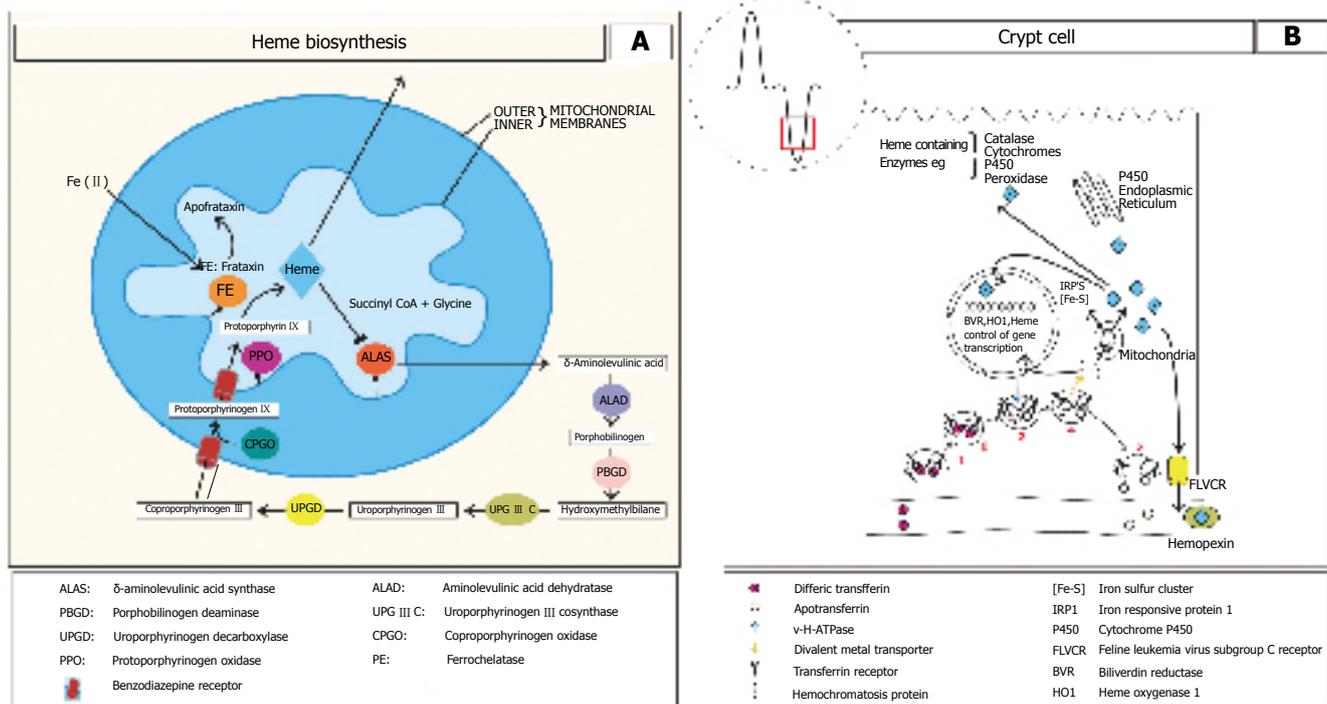


Figure 1 A: The heme biosynthetic pathway. Mitochondrial and cytosolic locations of the eight enzymes are shown circled and coloured. Commencing synthesis is ALAS on the inner mitochondrial membrane of the first intermediate as well as subsequent intermediates. Heme synthesis is regulated by heme at the level of ALAS via feedback repression. It has been suggested that frataxin may donate ferrous iron to protoporphyrin in the formation of heme; **B:** In the intestinal crypts the uptake of plasma transferrin-iron occurs by the transferrin receptor (TfR). In iron deficiency HFE complexes with TfR1 and to a much lesser extent with iron loading. (1) TfR binds to plasma diferric transferrin. (2) TfR is internalised by receptor mediated endocytosis. (3) In the cytoplasm a v-H-ATPase fuses with the endosome and acidifies it to release the iron from transferrin. Following ferrereduction Fe(II) is transported to the cytoplasm by a metal transporter. (4) possibly divalent metal transporter 1 (DMT1). The iron is then transported into the mitochondria where it is incorporated into heme. The mitochondria are also a major producer of iron sulphur clusters. (5) The transferrin receptor - apotransferrin complex then return to the cell membrane where at the neutral pH, apotransferrin dissociates. Heme, heme oxygenase and BVR may regulate gene transcription during enterocyte differentiation. FLVCR functions to export excess heme.

Function of HO

HO catalyses the mixed function oxidation of heme using cytochrome P-450, NADPH and molecular oxygen^[10-12]. HO functions in the oxidative cleavage of heme specifically at the α -methane bridge, resulting in the formation of biliverdin IX α which is rapidly reduced to bilirubin IX α by soluble biliverdin reductase (BVR). Since tissue BVR activity is 30-50 times greater than HO activity, this suggests that it is unlikely to limit heme breakdown, and that the rate limiting component is HO^[12]. Recently, the crystal structure of HO in complex with heme and biliverdin-iron has been solved^[13]. HO binds heme and oxygen between two helical folds with the proximal fold binding heme while the distal helix contains an oxygen binding site^[13].

Isoforms of HO

HO is expressed as two isoforms designated HO-1^[14] and HO-2^[14,15] which are products of different genes^[14]. HO-1 shares substantial homology with HO-2^[15]. The molecular mass of HO-1 is 32 kD, while HO-2 is 36 kD. HO-1 expression is induced by numerous factors, including oxidative stress, inflammation, cytokines, nitric oxide, prostaglandins, an elevated level of substrate^[16], iron deficiency^[17], metals including Cd, Co, Cr, Cu, Fe, Hg, Ni, Pd, Pt, Sn, Zn^[3,16,18,19], hyperoxia^[20] and UV light^[21]. The induction of HO-1 by hyperthermia has led to use of an alternate name, heat shock protein 32 (HSP-32)^[22]. Unlike

the inducible expression of HO-1, HO-2 expression is relatively constant.

HO and re-utilization of heme

HO-1 is mainly involved in the reutilization of heme-iron from hemoglobin and the expulsion of iron from tissue stores as evidenced by HO-1 knockout mice which develop anaemia because of progressive tissue iron retention particularly within macrophages^[23]. A previous study shows that less than 50% of endogenous hepatic heme degradation in rats is accounted for by HO-1 activity as evidenced by the generation of CO from heme^[24]. Therefore there appear two separate fates for catabolized heme-iron. Firstly a HO-1 dependent pathway, where iron from heme passes efficiently from the macrophage to the plasma, probably by the iron transporter ferroportin^[25], and secondly, a HO-1 independent pathway which results in retention of the freed iron.

HO and oxidative stress

HO-1 functions to diminish cellular oxidative stress because HO-1 reduces the levels of the pro-oxidant heme and produces the antioxidant bilirubin^[26]. Supporting this, humans deficient in HO-1^[27] and individuals with impaired transcription due to a microsatellite polymorphism in the HO-1 promoter region^[28,29] present with a phenotype similar to HO-1 knockout mice^[30]. Interestingly, HO-2 is unable to compensate for the loss of HO-1, probably

because its expression is restricted to a select group of cells or it is unable to be induced to the levels of activity required to produce the effects seen with HO-1 expression^[27-30]. HO-1 and intestinal oxidative stress is discussed in a later section.

INTESTINAL HEME BIOSYNTHESIS AND HEME OXYGENASE

Heme biosynthesis

The synthesis of heme and heme-containing proteins is crucial for intestinal function. These hemoproteins include electron carrying proteins such as cytochrome (CYP) P450 (see section on detoxification), mitochondrial localised cytochromes, the ferrioreductase Dcytb^[31], catalase and peroxidases which catalyse the reaction of hydrogen peroxide (H₂O₂) to water and oxygen (see section on oxidative stress). In addition to biosynthesis, heme can also be acquired by the enterocyte *via* intestinal absorption. This will be discussed in detail below with respect to the intestine.

HO gene expression

In the human intestinal cell lines CaCo-2 and HT-29, internalisation of heme increased HO-1 expression, indicating that the heme responsive element in the promoter region of the HO-1 gene was accessible and functional^[32,33]. Duodenal HO-1 expression is also increased in iron deficiency^[17] and by conditions that lead to oxidative stress including heavy metals and inflammation (see below with respect to the intestine). Up-regulation of HO-1 gene expression *via* the estrogen receptor β ^[34], octreotide, a somatostatin analogue^[35] and glutamine^[36] has been established. HO-2 expression is constitutive and mainly confined to the enteric nervous system and interstitial cells of Cajal, although it is possible that HO-2 is expressed by enterocytes^[37]. This will be addressed later in this review.

Heme turnover along the crypt-villus axis

Heme turnover is the balance between heme synthesis and its destruction by heme oxygenase. It is subject to variation along the crypt-villus length, being highest in the crypt and least at the villus tip^[38]. Thus the crypt region has the highest activity of both heme biosynthesis and heme oxygenase activity. As the cells migrate the rate of heme synthesis decreases but destruction decreases to a lesser extent, therefore total heme content is highest at the villus enterocytes compared with crypt epithelium.

HO-1 and intestinal cell proliferation and differentiation in the crypts

Cell turnover and differentiation is a function of crypt epithelium. Similar to that seen in the crypt epithelium, HO-1 activity is highest in undifferentiated intestinal epithelial Caco-2 cells^[39]. This suggests that HO-1 and cell proliferation/apoptosis may be linked^[40]. Supporting this, inhibiting HO-1 activity reduced cell proliferation and increased cell death^[40,41]. Conversely, in the human intestinal cell line HT-29 cells induction of HO-1 activity reduced expression of the pro-apoptotic gene caspase-3

and inhibited apoptosis. This supports the idea that HO-1 activity is anti-apoptotic^[42]. It is possible that HO-1 mediates these effects indirectly on gene transcription *via* the activity of BVR (Figure 2).

HO/BVR in intestinal cell signalling

BVR (EC 1.3.1.24) must undergo auto-phosphorylation in order to convert biliverdin to bilirubin^[43]. This property of phosphorylation/dephosphorylation during the conversion of biliverdin to bilirubin is similar to that seen with signalling kinases. Recent evidence indicates that BVR functions as a serine/threonine kinase that operates in the insulin receptor/MAPK pathways^[44] and a transcription factor with a bZip domain involved in ATF-2/CREB and HO-1 regulation^[45]. These additional roles suggest that BVR may have a broader function in regulating cellular activity^[46]. Since BVR immunoreactivity is seen in nuclei of epithelium lining the GI tract, this suggests a possible role in the regulation of gene transcription^[47].

HO-1 acts as a guardian of the genome during differentiation

It is possible that HO-1 may modulate proliferation by scavenging and/or preventing the formation of reactive oxygen metabolites (ROM) and reactive nitrogenous metabolites (RNM), since ROM inhibit Caco-2 cell proliferation^[48] and stimulate apoptosis^[49]. This is particularly relevant to the intestinal crypt region where proliferation exists and the levels of antioxidant detoxifying enzymes such as superoxide dismutase, glutathione peroxidase, glutathione reductase and catalase are low^[50]. If this is true then HO-1 level in the crypt region may act in defence against oxidative stress to limit mutation of DNA. HO-1 may therefore be one guardian of the genome, limiting mutations of DNA and promoting deletion of aberrant cells (Figure 2).

Differentiation is likely to result in elimination of cellular heme

As discussed previously the production of heme for enzymes, electron transport and as substrate for activity of HO1 and BVR is likely to be finely balanced since excess heme leads to oxidative stress and subsequent cell damage. Therefore as differentiation concludes heme production must fall. This may be achieved through reduced heme biosynthesis, increased HO-1 activity or increased heme export. With respect to heme export, a human heme exporter with homology to Feline leukaemia virus, subgroup C receptor (FLVCR) has recently been identified which has a clear function in erythropoiesis at the CFU-E stage of development^[51]. Impairment of FLVCR leads to the loss of CFU-E cells and impairs erythroid differentiation by inducing apoptosis. FLVCR is also expressed by Caco-2 cells, suggesting that it may be involved in intestinal differentiation by reducing the cellular heme concentration as the cell differentiates^[51]. This would reduce the oxidative burden on the stem/progenitor cell and potentially limit genomic damage^[52]. Supporting the existence of the FLVCR in the intestine, Caco-2 cells internalised heme by an active transport process and transcytosed it from apical to basal surfaces^[53].

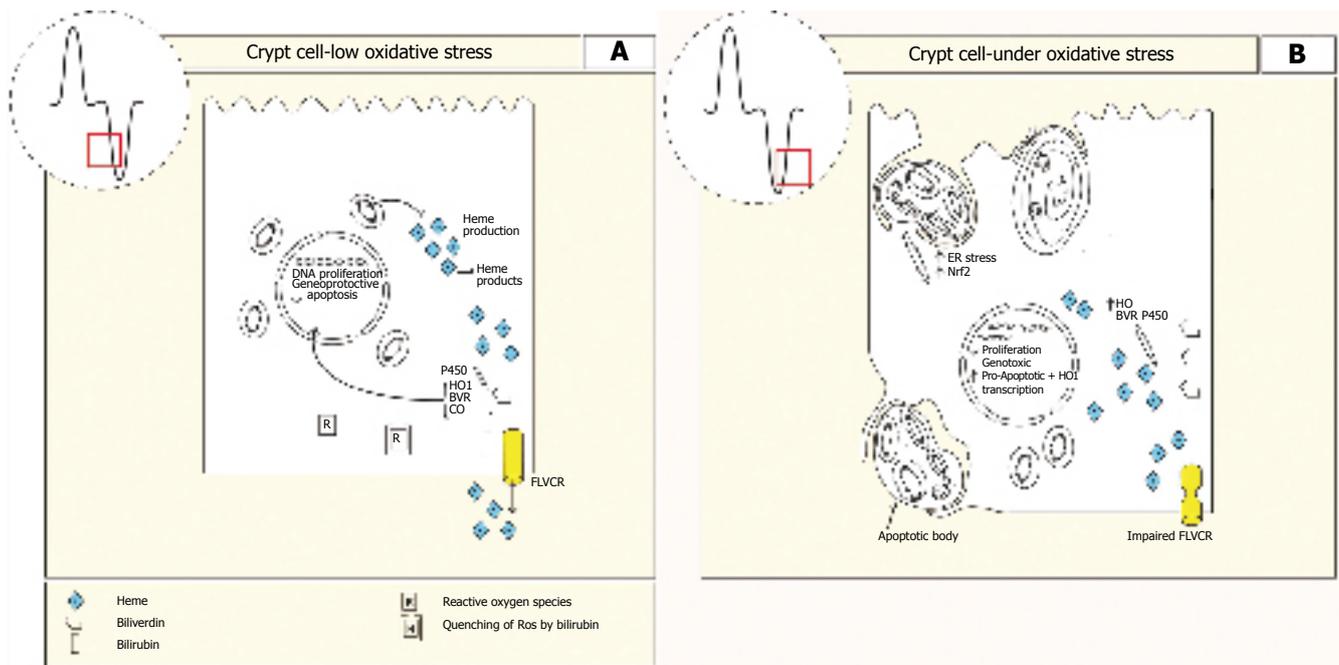


Figure 2 A: Epithelium of the crypt region is active in cell proliferation and differentiation. Heme production is required for the synthesis of heme containing enzymes. In these cells there are also high levels of heme oxygenase activity suggesting that heme breakdown is required for the production of bilirubin and carbon monoxide to maintain appropriate proliferation, differentiation and apoptosis. If the oxygen tension of the cell should increase or production of heme exceeds use, as would be seen as differentiation proceeds, then excess heme may be exported via FLVCR to limit oxidative stress. Increased oxidative stress may also be buffered by the antioxidant bilirubin; B: In the presence of increased oxidative stress caused by excess heme production, impaired FLVCR transport or increased oxygen tension, heme increases to levels that are genotoxic and the cell is predisposed to pro-apoptotic gene expression placing the cell into a death programme. Normal intestinal growth and differentiation would be impaired.

The converse was also true. Exposing the membranes to trypsin selectively increased the rate of uptake across the apical membrane only. Taken together these results raise the possibility that heme can be actively secreted from the cell in either direction possibly involving FLVCR (Figure 2).

HO activity along the length of the intestinal tract correlates with heme-iron absorption

HO activity is highest in the duodenum and lowest in the terminal ileum^[54-56]. This pattern of HO activity appears to correlate with the uptake of ingested xenobiotics and heme-iron absorption along the length of the intestinal tract (see below). In fact, treating rats with phenobarbital increased microsomal P450 enzyme activity, and absorption of iron from hemoglobin^[57]. Conversely, when an inhibitor of intestinal HO activity was given, intestinal heme-iron absorption decreased^[58] (see below).

HO and CYP450 activities in xenobiotic metabolism

The intestine makes an important contribution to the detoxification of many ingested xenobiotics (food additives, industrial chemicals, pesticides, plant toxins and pharmaceutical agents)^[59-61]. The heme containing P450 enzymes in particular the CYP3A superfamily are an integral component of xenobiotic detoxification. P450 levels are highest in the proximal duodenum, falling to lowest levels at the ileum^[62,63]. This correlates with the gradient of exposure to ingested xenobiotics. The highest activity of the P450 enzymes studied to date is the villus region^[64-67]. Interestingly, ingested xenobiotics induce greater CYP activity in the crypt epithelium compared with

villus enterocytes^[66]. Since the crypt cells do not absorb nutrients, this suggests that they passively absorb the drug or that the drug is actively absorbed by enterocytes and then taken up from the plasma by crypt cells. This interpretation is consistent with highest levels of heme biosynthesis in crypt epithelium.

Detoxification involves three phases, firstly the CYP450s and its mixed function oxidases adds a reactive group to the xenobiotic, secondly the molecule is made water soluble by conjugation to glucuronic acid, sulphates, glutathione or amino acids by UDP-glucuronosyltransferases [UGT], sulfotransferases [SULT] or glutathione S-transferases [GST], respectively, thirdly the metabolite is excreted from the enterocyte into the lumen by a transporter such as the ATP binding cassette transporters (ABC), P-glycoprotein^[59,62,63]. This "first pass" detoxification of xenobiotics is most active in the upper villus where absorption of nutrients and xenobiotics are greatest^[64-67].

To perform optimal detoxification the enterocyte must express appropriate levels of CYP450 and this is in part determined by heme turnover. Therefore for the enterocyte to express appropriate CYP450, adequate absorption of iron from the diet is required for heme synthesis along with conditions that limit HO-1 expression^[68-70]. If HO-1 activity is induced, for example by ingestion of environmental contaminants such as cadmium, organotin and heavy metals increased destruction of CYP will take place and first pass detoxification will be compromised. Similarly, iron deficiency reduces the ability to synthesise heme and therefore detoxify xenobiotics^[64,65,71]. This may

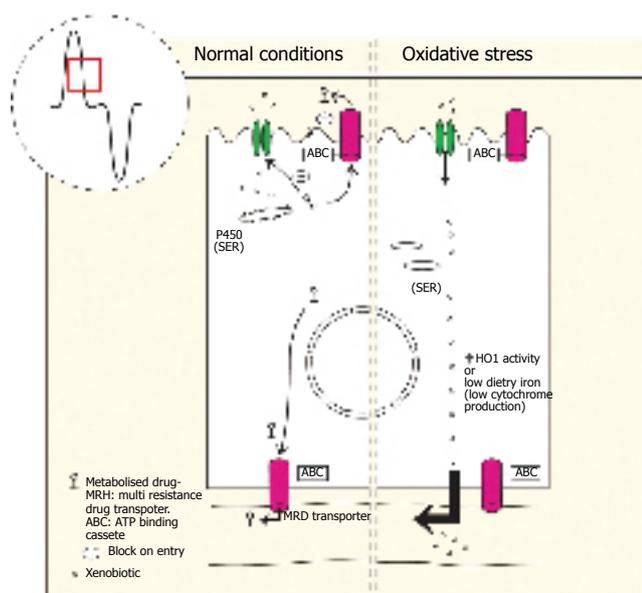


Figure 3 Left: Xenobiotics in the diet enter the enterocyte via facilitated diffusion or a specific transport process. Appropriate P450 expression on smooth endoplasmic reticulum (SER) enables first pass metabolism including phase I, and phase II metabolizing enzymes. Phase III multi drug resistance transporters (MDR) transport the conjugated-xenobiotic compound to the lumen or blood stream where increased hydrophilicity impairs re-entry into the enterocyte and leads to its elimination from the body directly. *De novo* synthesis of P450 occurs in the enterocytes and is dependent on appropriate levels of dietary iron. Right: In the presence of oxidative stress caused by high dietary intake of metals or compounds that induce heme oxygenase 1 (HO-1), heme containing P450 are broken down leading to increased entry of xenobiotics to the body. Dietary iron deficiency leads to reduced P450 activity and reduced detoxification capabilities.

therefore predispose an individual to cancer and ulceration of the colon^[72] (Figure 3).

HO and hyperbilirubinaemia

Several metalloporphyrins are competitive inhibitors of HO-1 activity because they have the capacity to interact with the heme binding site in HO-1, but are unable to activate the enzyme. This leads to a loss of heme degradation^[73-76]. This strategy has been used in the correction of human neonatal hyperbilirubinemia^[77-79]. Treatment with tin-protoporphyrin/mesoporphyrin, two structurally related heme analogues are effective in lowering serum bilirubin levels in many animals by competitively inhibiting HO^[73-79]. In addition, the use of short interfering RNAs targeting HO-1 mRNA expression has also been proposed to treat hyperbilirubinemia^[80]. Although there is a recognised loss of endogenous heme through the bile during metalloporphyrin administration^[81-83] that has been linked to an iron deficient state^[84], the iron deficiency has been shown to be readily reversible.

In the enterocyte, bilirubin is conjugated to glucuronic acid by bilirubin glucuronyl-transferase and excreted into the intestinal lumen^[85], or passed into the plasma where it non-covalently binds albumin and is transported to the liver, conjugated and excreted into the bile. However, early in perinatal life the luminal activity of secreted lysosomal-derived glucuronidase is high suggesting that enterocyte and biliary excreted conjugated-bilirubin can be deconjugated within the intestinal lumen enabling bilirubin to be reabsorbed *via* the enterohepatic circulation^[86]. This would contribute to neonatal hyperbilirubinemia.

Glutamine increases HO-1 expression

Glutamine is a major source of energy for the enterocyte and has been shown to promote intestinal growth and maintain intestinal integrity particularly when the intestine is heat stressed and starved^[36,87-91]. Glutamine stimulates intestinal proliferation and acts synergistically with epidermal growth factor to induce the mitogen-activated protein kinases and Jun nuclear receptor kinases. These in turn phosphorylate nuclear transcription factors such as AP-1 which activate transcription of target genes involved in cell proliferation and repair, including HO-1^[36,88]. Recently it was shown that glutamine stimulation of HO-1 expression was protective against endotoxic shock of the lower intestine^[90].

The inflammatory response and the role of HO-1

The epithelium lining the gastrointestinal tract presents a “mucosal barrier” to the migration of pathogens into the lamina propria that reside within the lumen of the gastrointestinal tract. In addition to the epithelium which is selectively permeable to nutrient absorption, the mucosal barrier comprises tight junctions that prevent migration of pathogens between cells. Breaching the mucosal barrier elicits an inflammatory response which first involves the innate immune system. Toll like receptors (TLR) expressed on the basolateral surface of enterocytes and the cell membrane of macrophages are activated^[92] and these in turn activate intracellular signalling pathways that induce NF- κ B dependent transcription of genes involved in the pro-inflammatory response such as cytokines, chemokines, immune receptors, nitric oxide synthase, prostaglandins and cell surface adhesion molecules^[93-95]. The pro-inflammatory mediators initially function to increase blood flow and edema. Concomitant with this, endothelial cell membranes express cell adhesion receptors including ICAM-1 that enable white blood cells to adhere and extravasate^[34]. The further release of pro-inflammatory chemokines (CINC-1, -3) may lead to hemostasis and organ failure^[34].

Inflammation is known to induce HO-1 gene expression and in turn its activity. The bilirubin and CO produced are thought to have restorative effects on impaired tissue function, in the case of bilirubin it is a potent anti-oxidant^[26,96-98]. There was increased oxidized bilirubin in the urine of patients following invasive surgery, supporting the idea that bilirubin acts as an antioxidant to scavenge reactive oxygen species^[97].

The second metabolite of HO-1 activity, CO has been shown to relax vascular smooth muscle by binding to the heme moiety of soluble guanyl cyclase (sGC). Activation of sGC increases blood flow to the site of intestinal injury^[99,100], inhibits platelet aggregation^[101], reduces microvascular fibrin accumulation^[102] and restricts leukostasis in postcapillary venules^[93,103]. Reduced leukostasis by CO is thought to occur *via* inhibition of the expression of the adhesion molecules, P-, E- selectins, and ICAM although some contribution by bilirubin is also thought responsible for the leukostasis^[104-106]. CO exerted additional cytoprotection by inhibiting components of the pro-inflammatory pathway including TNF- α , IL-1 β , IL-2,

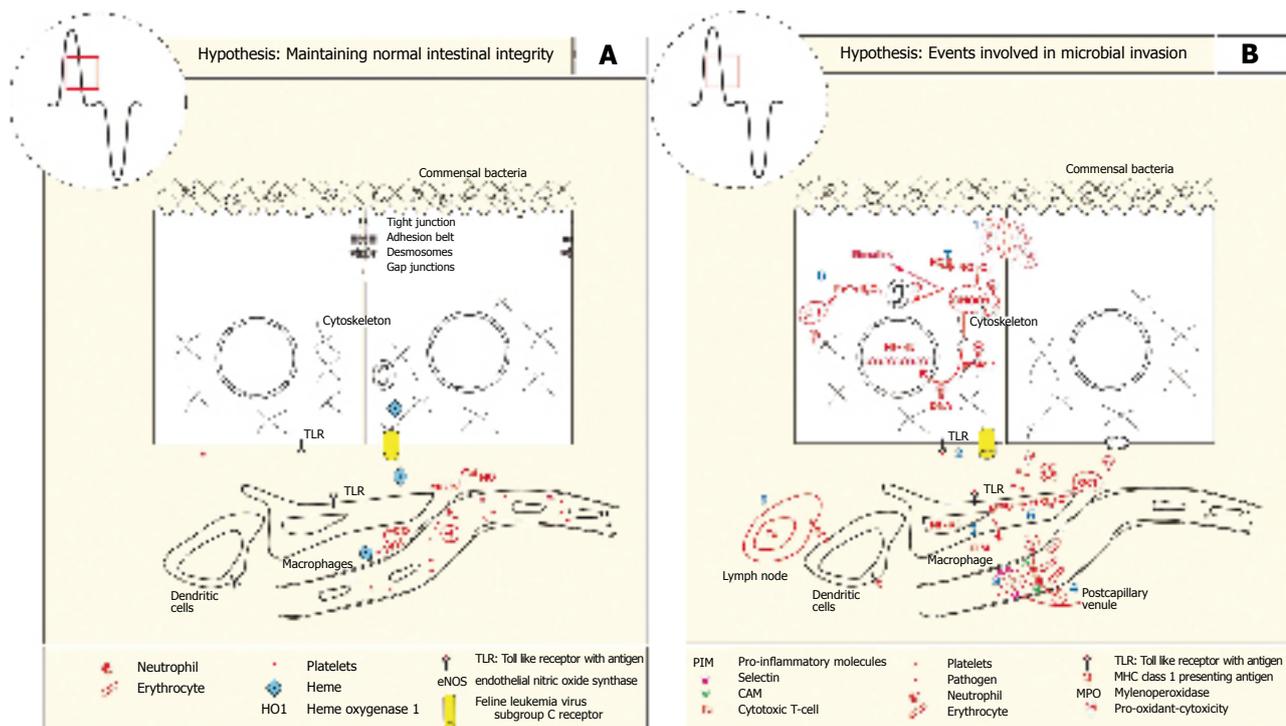


Figure 4 **A:** The intestinal mucosal barrier is maintained by a series of lateral membrane specialisations near the apical pole of the epithelial cell. It comprises tight junctions, adhesion belts, desmosomes and gap junctions that prevent the movement of pathogens across the epithelial monolayer. Constitutive expression and activities of endothelial nitric oxide synthase (eNOS) and heme oxygenase 1 (HO-1) is important for maintaining adequate blood flow, anti-inflammatory, anti-thrombotic (-) and anti-apoptotic effects on endothelium, neutrophils, platelets, and enterocytes, respectively. HO-1 activity produces the antioxidant bilirubin to limit oxidative damage; **B:** The loss of mucosal integrity results in the translocation of pathogens and establishment of an inflammatory response by the following series of events. (1) Initiation of synthesis of proteases by bacteria erode tight junction complexes between epithelial cells. (2) Binding of bacterial motifs activates toll like receptors, initiating the NF- κ B pathway. (3) Increased expression of pro-inflammatory cytokines, chemokines and endothelial cell surface adhesion molecules. (4) Leukocytes extravasate and increased permeability of capillaries increases fluid accumulation (5) Phagocytic cells produce myeloperoxidase which combines with peroxide to form hypochlorous acid that damages pathogen and host systems alike. (6) Peroxide produced by enterocytes in combination with ferrous iron can produce superoxide anions that damage lipids, DNA and proteins. (7) NO is produced at high concentrations that combines with peroxide to form the pro-oxidant peroxynitrite. (8) Platelets also bind to the endothelial surface to induce hemostasis. (9) Presentation of antigens by dendritic cells via major histocompatibility class 1 to cytotoxic T-cells leads to antibody presentation and destruction of infected epithelial cells.

IL-6, interferon- γ and cyclo-oxygenase, while stimulating the anti-inflammatory cytokine IL-10^[42,107-114] (Figure 4).

The third metabolite resulting from HO-1 activity is Fe(II). If this reaches the labile iron pool it will induce oxidative stress by participating in Fenton and Haber Weiss driven reactions and this would exacerbate inflammation. However, this is avoided by sequestration by ferritin^[21].

Endothelial nitric oxide synthase (eNOS) maintains mucosal integrity

Nitric oxide synthase (NOS) is a heme-containing enzyme that converts L-arginine to nitric oxide (NO) and citrulline. Similar to CO, NO binds the heme moiety of guanylate cyclase to produce vascular smooth muscle relaxation. Under normal circumstances eNOS/NO is important in maintaining mucosal integrity by modulating intestinal blood supply. NO at low concentrations stimulates mucous production, electrolyte secretion and decreases pro-inflammatory responses of mast cells, neutrophils, platelets and endothelial cells^[115-117] (Figure 4A).

Induction of nitric oxide synthase (iNOS) damages mucosal integrity

During inflammation cytokines activate NF- κ B dependent gene expression of iNOS by intestinal epithelial cells, neutrophils and macrophages. This leads to production

of NO^[116-118] at considerably higher levels than by eNOS activity. At this concentration NO reacts with superoxide anions to form the cytotoxic reactive nitrogen metabolite, peroxynitrite^[119-123]. Although peroxynitrite destroys micro organisms, it also reversibly inhibits heme containing proteins including cytochrome C, catalase, cytochrome P-450 and cytoskeletal proteins^[120,122]. It was suggested that inhibition of iNOS during endotoxin-induced gut mucosal dysfunction was beneficial because mitochondrial oxidative metabolism was unimpaired^[119]. This leads to maintenance of mucosal barrier integrity that resists bacterial translocation^[124] (Figure 4B).

Collectively, these findings indicate that at low concentrations NO maintains mucosal integrity, but at high concentrations NO induces reactive nitrogen metabolites which impair intestinal function.

The role of HO-1 versus iNOS in intestinal inflammation

During intestinal inflammation HO-1 mRNA expression increases in response to the activity of NO^[125]. It is likely that this is due to increased transcription and stabilization of existing transcripts^[125]. In addition, induction of HO-1 in a human intestinal cell line resulted in the degradation of cytokine-induced NOS. This reduced the production of NO and therefore peroxynitrite^[124]. Heme was also shown to reduce the NOS mRNA^[124]. The inhibition of NOS

activity by HO-1 was lost when tin protoporphyrin was given, indicating the direct effect of HO-1 in regulating NOS activity^[126]. These findings are consistent with a role for HO-1 in limiting the deleterious effects of excessive iNOS by directly inhibiting its transcription, degrading existing NOS and scavenging excess ROM/RNM with bilirubin.

NUTRITION AND MECHANISM OF HEME-IRON ABSORPTION

In western civilisations, 40% of the average non-vegetarian person's total body iron is derived from heme products. However, iron from these substances only constitutes 15% of ingested iron^[127,128], suggesting that heme-iron is more efficiently absorbed than non-heme iron. This observation also explains why vegetarians are more prone to iron deficiency than meat eaters. Despite the importance of the contribution of heme to body iron stores, how it is absorbed is still poorly understood.

Mechanism of Heme-Iron Absorption

It is generally recognised that in omnivorous animals, heme is not transferred into the blood as an intact metalloporphyrin, instead absorption of iron from heme involves three steps (1) Uptake of luminal metalloporphyrin [Fe(II)-protoporphyrin-IX] by the enterocyte (2) catabolism within the enterocyte, combining of pools of ingested iron from non-heme and heme sources and (3) release of elemental iron to the bloodstream by the enterocyte^[129-133]. A large number of proteins are thought to be involved in the mechanism of heme iron absorption and these are tabulated along with their sites of expression and function (Table 1). Most of these proteins will be discussed individually in the following sections and is also summarised in Figure 5.

Worthington and co-workers used immunofluorescent methods to show that the uptake of a heme analog was temperature and time dependent, could be inhibited by heme competition and augmented by inhibitors of heme synthesis^[134]. It is likely that Worthington and co-workers identified a heme transport process by Caco-2 cells that may be a transporter and/or possibly a heme receptor.

Heme uptake by a heme transporter

Heme is taken into the enterocyte intact as evidenced by the recovery of ⁵⁹Fe-heme from the small intestinal mucosa following the gavage of radiolabelled hemoglobin^[130-133]. This process is energy dependent indicating an active process^[135]. The finding that absorption of iron from hemoglobin and heme were equivalent suggests that uptake of heme is independent of the redox state of the heme-iron^[136,137]. Alternatively there is an oxidoreductive mechanism on the cell surface that is capable of converting the iron redox state before internalization.

A microvillus membrane transporter that imports heme from the lumen into enterocytes of mice was recently characterised^[138]. This protein was expressed in the duodenum but not the ileum, consistent with expression at the site of highest heme-iron absorption. Heme carrier

Table 1 Proteins involved in intestinal heme-iron absorption along with their function, location and whether they are regulated by iron

Protein	Function	Location	Regulation by Fe
Heme receptor	Receptor for heme	?	Inversely
HCP1	Transporter of heme	AM -> BC	Constant
FLVCR	Heme exporter	?	Unknown
Ferritin	Iron storage	C	Directly
DMT1	Fe(II) importer	AM+Lys	Inversely
Ferroportin	Fe(II) exporter	BL AM	Inversely
Hephaestin	Ferroxidase + ?	SN, BL	Constant
HO 1	Degradation of heme	C	Inversely
HO 2	Degradation of heme	SMC, EN	Constant
HFE	Regulator	TW	Inversely
TfR1	Tf:Fe endocytosis	BL, SN	Constant
Transferrin	Endosomal iron transport	C	Inverse

DMT1 = divalent metal transporter 1; HO = heme oxygenase; HCP1 = heme carrier protein 1; FLVCR = Feline leukaemia virus, subgroup C receptor; HFE = hemochromatosis protein; TfR1 = transferrin receptor 1; AM = apical membrane; BL = basolateral membrane; SN = supranuclear; LM = lateral membrane; Lys = Lysosomes; TW = terminal web; C = cytoplasm; BC = basal cytoplasm; SMC = smooth muscle cells, EN = enteric nerves; ? = putative; Tf:Fe = transferrin iron.

protein 1 (HCP1) encodes a protein with strong homology to bacterial tetracycline-resistance proteins, which are characterised as having 12 transmembrane domains and are members of the major facilitator superfamily^[138]. Functional characterisation of HCP1 using *Xenopus* oocytes revealed selectivity for the transport of heme but not tetracycline or non-heme iron. *In vitro* studies involving HCP1 siRNA and *in vivo* studies blocking HCP1 activity by antibodies indicated that the uptake of heme fell. HCP1 also required energy but the source of energy is presently unknown. Collectively, these findings indicate the first functional characterisation of a heme specific transporter.

Interestingly, during conditions known to increase non-heme iron absorption such as hypotransferranemia and iron deficiency, HCP1 mRNA expression remained constant although it was increased by hypoxia. Similarly, the extent of HCP1 protein expression remained constant with respect to the iron content of the enterocyte, although the protein translocated from the microvillus membrane to the basal cytoplasm during iron loading. The lack of increased expression of HCP1 by iron deficiency may in part explain the limited ability to increase heme-iron absorption. It may also indicate that HCP1 needs additional modulating proteins in order to regulate heme-iron absorption (Figure 5).

Heme uptake by a heme receptor

Previous studies have reported a 50% increase in heme binding to microvillus membrane preparations during iron deficiency, raising the possibility of a brush border localised heme receptor^[139-142]. This is based on the measurements of binding [¹⁴C]-heme to semi-purified brush border preparations^[139-142]. Subsequent solubilisation of the brush border microvillus membranes identified

the size of the heme binding substances, one with a molecular mass of about 250 kDa the other about 60 kDa. Displacement of the [^{14}C]-heme by unlabelled heme was seen with the 250 kDa complex, but not the about 60 kDa complex^[139-141], suggesting the larger peak represented a heme receptor complex, while the smaller peak was thought to be polymerised heme^[140]. Based on the capacity of the large complex to be saturated with heme and having an K_a of 10^{-6} to 10^{-7} mol/L this suggests that it is a relatively high affinity heme receptor.

In addition to the identification of a putative heme receptor in the intestine, others have identified a heme binding protein that is distinct from the hemopexin receptor^[143] with similar binding characteristics to the intestinal heme receptor. Since the heme binding protein and HCP1 have molecular weights of about 250 kDa and about 50 kDa, respectively, it is unlikely they are the same protein, unless HCP1 forms part of a larger complex. The finding that erythroleukaemic cells internalise heme coated latex beads^[144,145] and that trypsin treatment eliminates heme binding^[146,147] supports the existence of a heme receptor-mediated, endocytotic pathway. It therefore appears that there are at least two defined pathways involved in the uptake of heme into the enterocyte, one involving HCP1^[138] and the other a receptor-mediated endocytotic process^[139-142,144-147]. Despite considerable characterisation of the heme receptor almost thirty years ago there has been little progress made since (Figure 5).

Intracellular processing of heme

Morphological studies show that following ingestion of a heme-rich meal by rodents, heme was first seen along the microvillus membrane, then in tubulovesicular structures of the apical cytoplasm and finally in secondary lysosomes^[148,149]. Based on time course studies, DAB (3,3-Diaminobenzidine tetrahydrochloride) disappeared from lysosomes suggesting that heme was either transported from these structures or that it was degraded within them. In either case heme degradation involves HO activity but whether this is HO-1 or HO-2 is presently unknown.

Alcohol and heme-iron absorption

In rats treated with alcohol there was increased absorption of iron from heme as well as the entire hemoglobin complex where it was transported to the liver as a haptoglobin-hemoglobin complex^[150,151]. Thus, absorption of iron from hemoglobin also appears to contribute to the iron over loading caused by excessive alcohol consumption.

Limitations in iron absorption from heme

The intracellular sites where restrictions to the absorption of iron from heme occur have been studied in dogs given radiolabelled hemoglobin and then measuring the progression of radioactivity through mucosal compartments^[133]. The most likely sites where the rate of iron absorption was limited appears to be at the stage of heme breakdown and/or the release of iron from the cell. This might involve the steps where HO operates, where iron is released out of an intracellular compartment, or from the cell (see below).

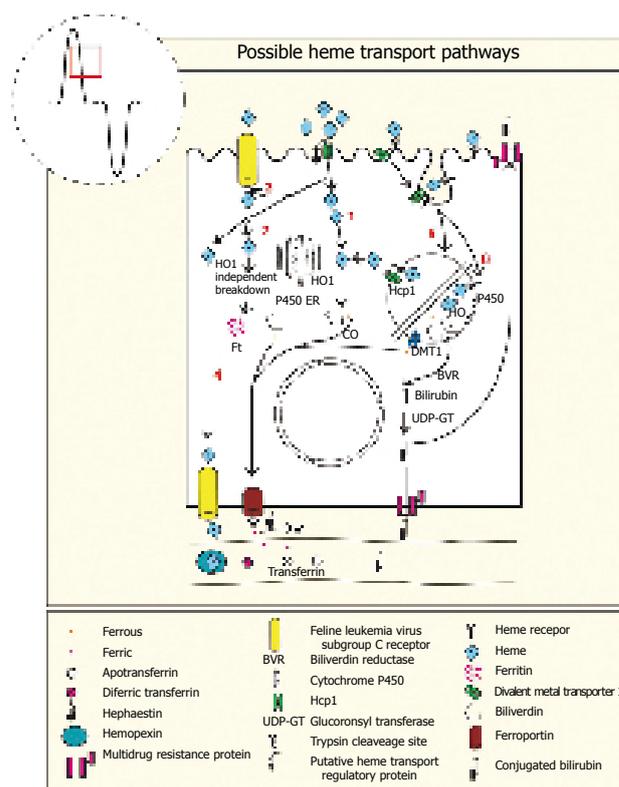


Figure 5 Six steps in the uptake of heme by intestinal enterocytes. Heme taken up by heme carrier protein (HCP1) is internalised and broken down in the cytoplasm by HO-1 (1), by a HO-1 independent enzyme(s) (2), some is released intact back into the lumen (3) or plasma by FLVCR (4). Heme may also bind to a heme receptor and with HCP1 be internalised by tryptase mediated endocytosis. The heme may be released to the cytoplasm by HCP1 (5), or the heme may be broken down in the lysosome and the released iron transported to the cytoplasm by the divalent metal transporter (DMT1) (6). The iron released from heme passes to the basal cytoplasm and is transported across the basal membrane by ferroportin in the ferrous state, oxidized to ferric-iron by hephaestin and transported in the plasma by transferrin.

Other proteins possibly involved in the transport of Fe(II) from heme

In view of the likely convergence of iron derived from sources of non-heme and heme iron what is known for non-heme iron is described.

Divalent Metal Transporter 1 (DMT1)

The Microcytic mouse (*m κ*) and anaemic Belgrade rat (*b*) have an autosomal recessive inherited, hypochromic, microcytic anaemia associated with a well-characterised defect in the transferrin cycle in erythroid cells^[152], as well as a defective intestinal non heme-iron transport that is manifest at the site of uptake at the microvillus membrane^[153]. The similar phenotypes are explained by an identical mutation in DMT1 at G185R^[154,155]. Deletion of DMT1 also resulted in loss of iron transport by the intestine but not the liver or placenta^[156]. The finding that heme is broken down intracellularly and a portion of DMT1 is found inside the enterocyte could suggest that DMT1 is involved in heme-iron absorption. There is an absolute requirement for DMT1 in the uptake of iron by the intestine^[156], suggesting that intestinal absorption of iron from heme also requires DMT1 but this remains to be determined (Figure 5).

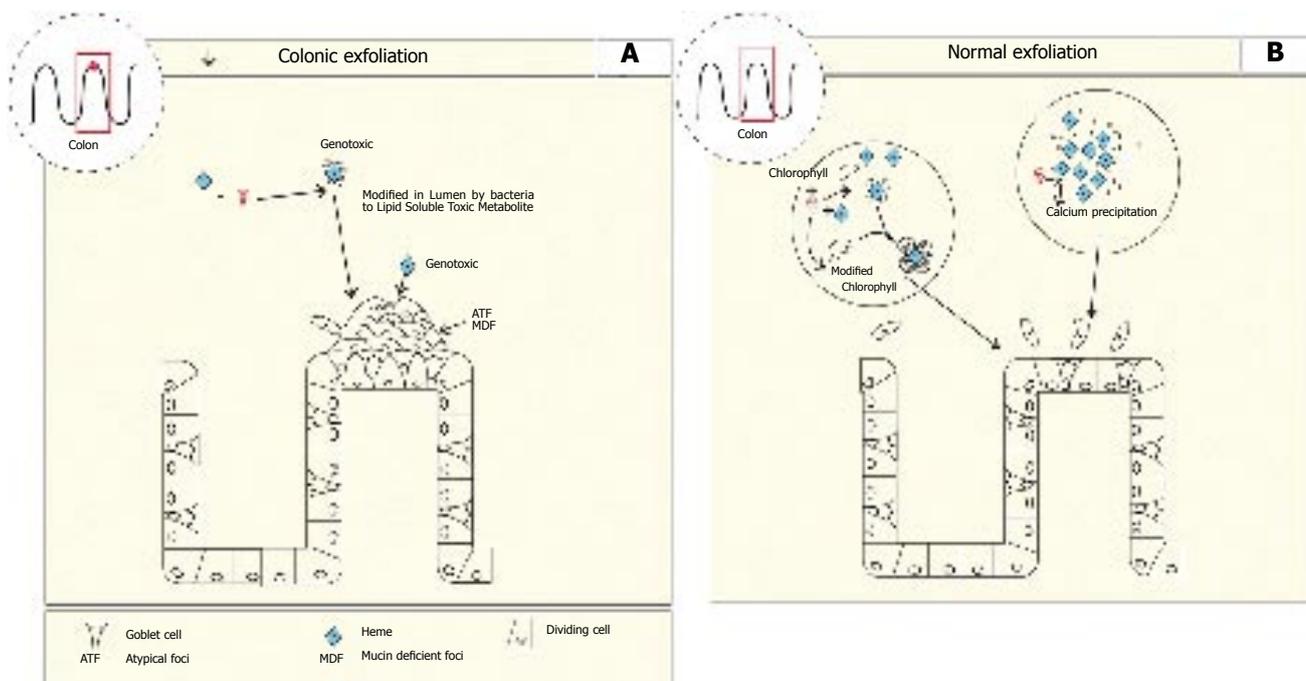


Figure 6 A: In the colon excess heme is metabolised into a lipid soluble heme metabolite possibly by commensal bacteria. Heme itself is also genotoxic. This results in the formation of aberrant atypical foci, that are mucin deficient (ATF, MDF). Apoptosis is inhibited which could lead to increased survival of mutant cells; **B:** In the presence of calcium or chlorophyll heme precipitates into biological inactive compounds which inhibit the heme factor or binds the heme factor rendering it inert, respectively leading to normal colon growth.

Hemochromatosis protein (HFE)

Intestinal expressed HFE is recognised to regulate iron absorption *via* the uptake of transferrin bound iron by crypt cells. The finding that HFE is expressed along the terminal web of enterocytes during iron deficiency where it co-localised with DMT1, raises the possibility that HFE may function directly in iron absorption and this may include heme-iron^[157]. This is also supported by the finding that HFE expression is inversely proportional to iron absorption^[157]. If this is the case then HFE is positioned to interact with HCP1, the putative heme receptor and DMT1. Whether DMT1 and HFE work intracellularly (such as in lysosomes) at levels that cannot be detected by immunofluorescent microscopy remains to be determined.

Ferroportin

Basolateral transport of non-heme iron involves ferroportin/Ireg-1/MTP-1/SLC40A1, most often referred to as ferroportin^[25]. This is based on the study showing that over-expression of ferroportin in macrophages during erythrophagocytosis increased release of non-heme iron, but not heme^[158]. This observation is likely to apply to the enterocyte but this needs to be determined. Also selective deletion of ferroportin in mice resulted in non-heme iron accumulation within enterocytes^[159] which provides support for the hypothesis that ferroportin functions with non-heme iron (Figure 5).

Mammalian iron-ATPase

Baranano and co-workers have identified a microsomal membrane Fe(II) transporter from the spleen which presumably represents an iron transporter expressed by macrophages. It is induced by heme, and depends

on hydrolysable triphosphate, magnesium and temperature^[160]. It is proposed that following heme catabolism by macrophages, Fe(II) is shunted into the lumen of the endoplasmic reticulum. Others have found a similar transporter in liver microsomes^[161]. Whether this transporter functions in enterocytes remains to be determined.

HEME AND COLON CARCINOGENESIS

Although heme-iron is more bio-available than non-heme iron it has limited ability to be absorbed. Therefore, unabsorbed heme reaches the colon. Luminal heme is also derived from the blood *via* extravasation and from desquamation. Previous studies have shown that heme irritates the epithelium of the colon as evidenced by mild diarrhoea^[162,163]. It was shown that feeding heme but not non-heme iron to rats results in significant increased proliferation of colonic mucosa^[162]. In addition, the incidence of aberrant atypical foci (ATF) and mucin-depleted foci (MDF)^[164] increased as the heme content of the diet increased suggesting that heme is carcinogenic^[164,165]. In fact, it was demonstrated that heme was genotoxic in the human colon tumour cell line HT29^[166].

It has been shown that a heme breakdown product rather than heme or iron *per se* was responsible for the inflammation and ATF formation^[162,163]. In the colon some heme breakdown products are produced by the presence of colonic bacteria^[167], and it has been suggested the heme is converted to a cytotoxic factor, although it has not been fully characterised^[162,163]. Gene microarray analysis of 365 genes expressed by the colon revealed that feeding heme

down-regulated mucosal pentraxin 30-fold^[168,169]. Since pentraxin is involved in the recognition and clearance of dying cells, a process that is normally ongoing in the intestinal tract, downregulation of this gene by heme infers that apoptosis of colonic mucosal cells may be inhibited. If this is true then it might explain the increased carcinogenic potential if cells with mutated genomes cannot be eliminated^[168]. In support of this, De Vogel et al., showed that heme supplementation decreased colonic exfoliation^[170] (Figure 6).

The cytotoxic affect of heme on the colon was lost when the diet was supplemented with green vegetables^[170]. It was hypothesised that chlorophyll in green vegetables inhibited the formation of the heme factor by competing for solubilisation with heme in the large intestine. Alternatively, chlorophyll and heme could form a complex that blocks the site of covalent modification of the heme and reduces the formation of the heme factor^[170]. Calcium was also shown to protect against the effects of heme on colonic proliferation and normalising pentraxin expression, presumably because calcium precipitates heme, thereby preventing the formation of the soluble heme induced cytotoxic factor^[169,171,172]. This conclusion is consistent with the inhibitory effect that calcium has on heme bioavailability for its absorption in the small intestine^[171] (Figure 6A and B).

HEME AND HO-2 IN INTESTINAL MOTILITY

Peristaltic contractions are controlled by stellate shaped non-neuronal interstitial cells of Cajal (ICC) situated within the myenteric plexus (ICC-MY)^[173-177]. Clusters of spindle shaped bipolar ICC found throughout the circular and longitudinal muscle layers (ICC-DMP) generate pacemaker potentials spontaneously but these are modified by neural input^[177]. Adjacent to the submucosa and within the circular muscle layer ICC also appear to synapse with nerves (ICC-IM)^[174-177]. Loss of ICC leads to markedly impaired neurotransmission and typical gastrointestinal motor patterns indicating their importance in coordinating neural modulation of intestinal motility. In the small intestine ICC-MY appear important for pacemaker ICC but in other regions of the bowel this is regulated by ICC-IM.

The network is connected to the smooth muscle syncytium *via* either gap junctions or peg in socket junctions. These membrane specialisations provide a means of conducting pacemaker currents to intestinal smooth muscle^[174-177]. It is thought that pacemaker potentials originate from unitary potentials caused by the release of calcium from mitochondrial stores^[177,178] which in turn cause a rise in membrane potential generated by opening of Ca²⁺ permeable channels. The plateau component observed in pacemaker potentials is generated by opening Ca²⁺ activated Cl⁻ channels^[179]. Repolarisation involves removal of cytosolic Ca²⁺ to stores and K⁺ transport *via* activated K⁺ channels^[179]. The frequency of these events establishes the pacemaker potential of a particular region of the intestine. Muscle contraction will occur providing the membrane potential is capable of activating L-type

Ca²⁺ channels and depolarising the cell^[180]. The resulting increase in cytosolic Ca²⁺ levels is coupled to contraction. Contraction is limited by activation of large-conductance Ca²⁺-activated K⁺ channels and L-type Ca²⁺ inactivation^[180].

It has been shown that HO-2 but not HO-1 is present in all classes of ICC (-MY, -IM & -DMP), although HO-2 expression was greater in ICC-MY than in ICC-IM. Enteric neurons also express HO-2^[180-192]. In the gastric fundus and in particular mucosal epithelial cells, neurons of the submucosal and myenteric plexus and ICC co-express HO-2 and BVR indicating that these cells have the capacity to generate bilirubin^[47]. Since ICC have numerous mitochondria it is hypothesised they produce heme to serve as substrate for HO-2 activity and the CO produced may regulate membrane potential and in turn affect intestinal contraction^[186]. In the genetic absence of ICC and in HO-2 knockout mice the membrane potential of intestinal smooth muscle is depolarised compared with wild type controls^[174,185,188]. Studies have shown that the HO-2 mediated hyperpolarisation is probably due to the effect of CO on activation of K⁺ currents in smooth muscle^[181,184], and that exogenous CO given to HO-2 knockout mice hyperpolarises the resting membrane potential^[191]. Supporting this, the membrane is more hyperpolarised near the submucosa and these cells have higher HO-2 activity and CO production than cells near the myenteric plexus where the membrane is more depolarised^[191]. Taken together it suggests that CO produced from ENS and ICC function in maintaining membrane potential and the gradient that exists along the longitudinal and across the circular musculature^[184,191]. It would be expected that increased CO production would result in a greater level of smooth muscle relaxation because the membrane potential is further away from threshold. The mechanism by which CO reduces the resting membrane potential is unclear^[188].

CONCLUSIONS

Within the intestine heme serves important roles in energy production, in enzymes involved in detoxification, in the generation of the second messenger gases NO and CO and the antioxidant bilirubin. The products of heme breakdown namely CO and bilirubin restrict oxidative stress, inflammation, and regulate the cell cycle and differentiation in the crypt region. Excess heme may also promote the development towards colon cancer. Dietary heme is an important source of iron for the body and the absorption of iron from heme differs from non-heme. The molecular mechanism operating in the early phases of absorption appears to involve a transporter although there is evidence of a receptor mediated process and numerous other proteins may function in heme-iron as in non-heme iron absorption. The ability of HO to perform these varied functions within the enterocyte probably depends on different compartments within the cell which are differentially accessed by heme and HO. Future studies will determine how heme-iron is absorbed and the mechanisms by which HO regulates the cell cycle and differentiation, limits the inflammatory process.

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Nitrosamine and related food intake and gastric and oesophageal cancer risk: A systematic review of the epidemiological evidence

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CONCLUSION: The available evidence supports a positive association between nitrite and nitrosamine intake and GC, between meat and processed meat intake and GC and OC, and between preserved fish, vegetable and smoked food intake and GC, but is not conclusive.

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Key words: Nitrites; N-nitrosodimethylamine; Nitroso-compounds; Dietary intake; Gastric cancer

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Abstract

AIM: To study the association between nitrite and nitrosamine intake and gastric cancer (GC), between meat and processed meat intake, GC and oesophageal cancer (OC), and between preserved fish, vegetable and smoked food intake and GC.

METHODS: In this article we reviewed all the published cohort and case-control studies from 1985-2005, and analyzed the relationship between nitrosamine and nitrite intake and the most important related food intake (meat and processed meat, preserved vegetables and fish, smoked foods and beer drinking) and GC or OC risk. Sixty-one studies, 11 cohorts and 50 case-control studies were included.

RESULTS: Evidence from case-control studies supported an association between nitrite and nitrosamine intake with GC but evidence was insufficient in relation to OC. A high proportion of case-control studies found a positive association with meat intake for both tumours (11 of 16 studies on GC and 11 of 18 studies on OC). A relatively large number of case-control studies showed quite consistent results supporting a positive association between processed meat intake and GC and OC risk (10 of 14 studies on GC and 8 of 9 studies on OC). Almost all the case-control studies found a positive and significant association between preserved fish, vegetable and smoked food intake and GC. The evidence regarding OC was more limited. Overall the evidence from cohort studies was insufficient or more inconsistent than that from case-control studies.

INTRODUCTION

Humans are exposed to a wide range of N-nitroso-compounds (NOCs) from diet, tobacco smoking, work place and drinking water^[1,2], which are the major source of exposure in the general population^[3]. Preformed exogenous nitrosamines are found mainly in cured meat products, smoked preserved foods, foods subjected to drying by additives such as malt in the production of beer and whiskey, pickled and salty preserved foods^[2]. Available data suggest that nitrosamines are found more frequently and at higher concentration in Asian foods than in Western foods^[4]. On the other hand, nitrosamines are formed endogenously from nitrate and nitrite. Although the levels have reduced during the last 20 years, sodium nitrites are still widely used as food preservatives in cured meat products. Nitrite is also formed in the human body from oral reduction of salivary nitrate. Vegetables and water are the main sources of nitrate intake. Nitrites are transformed into nitric oxide by gastric acid-catalysed formation, which acts as a nitrosating agent of amines and amides, as consequence of NOC^[2]. Under chronic inflammatory conditions, such as precancerous conditions of gastric cancer (GC) and oesophageal cancer (OC), nitrosating agents are overproduced^[1]. Studies in volunteers have shown that red meat intake has a consistent dose response in the endogenous formation of NOC measured in faecal samples, while white meat intake has no effect^[5].

So far, there is no conclusive epidemiological evidence that nitrosamines are carcinogenic to humans, although they produce a wide range of tumours in more than 40 animal species tested^[6]. Two important nitrosamines, namely N-nitrosodiethylamine (NDEA) and N-nitrosodimethylamine (NDMA), are classified as probably carcinogenic to humans (group 2A) by International Agency for Research on Cancer (IARC)^[7]. One previous comprehensive review on nutrition and cancer^[8] concluded that there is convincing evidence that the consumption of the Chinese salted-dry fish is causally associated with the risk of nasopharyngeal cancer with their nitrosamine content being the most plausible agent. Evidence of an increasing cancer risk due to N-nitrosamine and cured meat intake is considered insufficient for GC and possible for OC^[8]. A previous review of dietary nitrates, nitrites and NOC and risk of nasopharynx, oesophagus, stomach, pancreas, colorectal and brain cancer concluded that epidemiological evidence related to GC and other tumours remains inconclusive, although the strongest evidence pointed to an increased risk of nasopharyngeal and oesophageal cancer in subjects exposed to high dietary NOC levels^[9].

Several foods, such as processed meat and dried salted fish, which are sources of nitrites and/or nitrosamines, are also important sources of salt. Salt produces an inflammatory process leading to damage of the protective stomach mucosa and increases the risk of stomach cancer^[8]. *H. pylori* infection may be related to salt and NOC, in enhancing carcinogenesis after the epithelium is damaged^[8].

The aim of this article is to review and evaluate the available epidemiological evidence about the association between dietary exposure to preformed nitrosamine and related food intake and gastric and oesophageal cancer risk in humans.

MATERIALS AND METHODS

Inclusion criteria

Epidemiological studies (case-control or cohort studies) published between 1985 and 2005 evaluating the relationship between nitrosamines, NDMA, nitrites, food sources of exogenous and endogenous nitrosamines, and oesophageal or gastric cancer risk in males and females were included in the study. Experimental studies were not considered.

Search strategy

We conducted electronic searches in MEDLINE and CANCELRIT databases from 1985-2005. The search strategy included the following terms “oesophageal”, “gastrointestinal” “gastric”, “stomach”, “upper aero digestive tract”, “cancer”, “nitrosamines”, “NOC”, “NDMA”, “processed meat”, “meat”, “intake”, “salted fish”, “dietary patterns”, “nitrites” and “diet”. The search was supplemented with references included in recovered papers that were not identified in the electronic search. References contained in recent reviews of the literature were also consulted^[10].

STUDIES

GASTRIC: COHORT

- ¹Knekt^[11] (1999)-Finland
- ²Knekt^[11] (1999)-Finland
- ²Van Ioon^[12] (1998)-The Netherlands

GASTRIC: CASE-CONTROL

- ¹Risch^[13] (1985)-Canada
- ¹La Vecchia^[14] (1995)-Italy
- ¹De Stefani^[15] (1998)-Uruguay
- ¹Pobel^[16] (1995)-France
- ³Gonzalez^[17] (1994)-Spain
- ²La Vecchia^[14] (1995)-Aital
- ²Pobel^[16] (1995)-France
- ²Buiatti^[18] (1990)-Italy
- ²Gonzalez^[17] (1994)-Spain
- ²La Vecchia^[14] (1997)-Italy
- ²Mayne^[19] (2001)-USA
- ²Risch^[13] (1985)-Canada

OE SOPHAGEAL: CASE-CONTROL

- ²Mayne^[19] (2001)-USA
- ²Rogers^[21] (1995)-USA
- ¹Rogers^[21] (1995)-USA

OR (95% CI)

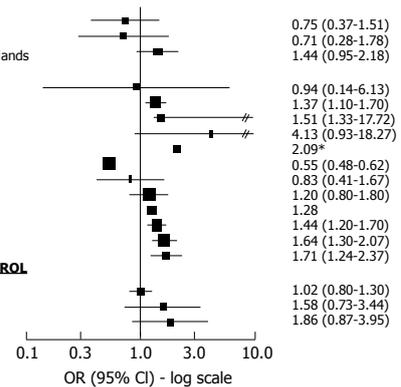


Figure 1 Nitrites and nitrosamines intake and gastric or oesophageal cancer. *Without confidence intervals, but statistically significant. ¹Ndma; ²Nitrites; ³Nitrosamines.

Data extraction

The following information was gathered from the original publications: study data (author, journal, year, country); epidemiologic design including type of study, number of subjects, follow-up (years), number of cases/controls, type of controls; diet including type and quality of dietary assessment method, number and type of food items; results including the most fully adjusted odds ratio or rate ratios and 95% confidence intervals for the highest and lowest categories of compound/food intake used from each included article. Covariates included in the analysis were also evaluated.

Exposure definition and classification

Since information about gastric cancer and NOC and their precursors was heterogeneous, sources of exposure were classified into two groups: nitrosamines and nitrites; food sources of endogenous (red meat) and exogenous (processed meat, beer, pickled and dried vegetables, smoked fish or meat, salted and dried fish or meat) nitrosamines. The odds ratio for each study was plotted in the included Figures, using symbols whose size was proportional to the study size.

RESULTS

Study characteristics

A total of 75 publications potentially eligible for inclusion in this review, were identified. After a detailed examination, in which some papers with duplicate or inappropriate information were detected and excluded, 63 studies were finally selected^[11-71,78-80]. Of these, 52 studies were case-control studies and 11 were cohort studies. Most of them were carried-out in Asia (35%), Europe (30%), and USA (23%).

Dietary intake of nitrosamines or nitrites

Cohort studies: We found 2 cohort studies^[11,12] with information on GC and nitrites, nitrosamines or both (Figure 1). In relation to nitrites, one found a positive but not significant association^[12] while the other found

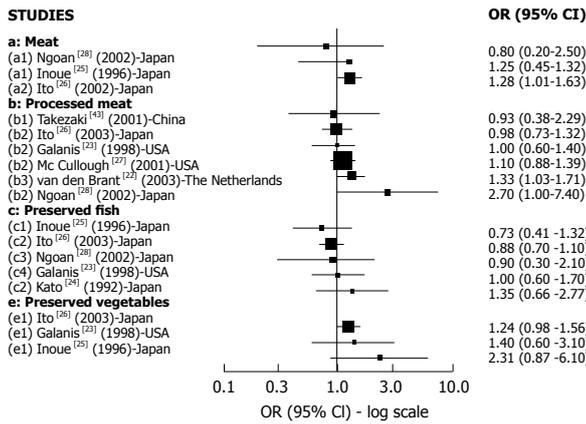


Figure 2 Meat, processed meat, preserved fish and preserved vegetables and gastric cancer (cohort studies). (a) Meat intake: (a1) meat (a2) pork; (b) Processed meat: (b1) salted meat (b2) processed meat (b3) bacon; (c) Preserved fish: (c1) salted/dried fish (c2) salted fish (c3) processed fish (c4) dried fish; (e) Preserved vegetables.

no association^[11]. Only one cohort study investigated the association between NDMA and GC and found no association^[11]. We did not find any cohort studies investigating the relationship between nitrosamine or nitrite intake and OC.

Case-control studies: We found 8 case-control studies with information on GC and nitrites, nitrosamines or both^[13-20] (Figure 1). Among the 7 studies on nitrites and GC^[13-17,19,20], 5 showed a positive association^[13,15-17,20] and 3 achieved statistical significance^[15-17]. Two of them^[15,16] were large studies, adjusting for all relevant confounding factors. In relation to nitrosamine intake and GC, among the 5 studies published^[14,17-20], 4 found a positive association which was statistically significant (SS) in 3 of them^[18-20]. We found only 2 case-control studies reporting results in relation to OC which showed no association with nitrite intake^[16] or no significantly positive association with nitrite and NDMA intake^[21] (Figure 1).

Dietary intake of food sources of exogenous and/or endogenous nitrosamines

Cohort studies: We found 8 cohort studies with results about GC risk and food sources of exogenous nitrosamines and/or foods than could enhance their endogenous formation^[22-28,43] (Figure 2). Only 3 studies reported results in relation to red meat intake. A positive and statistically significant association was observed with pork in one large study^[26] while no association was found in the other studies with few GC cases^[25,28]. In relation to high processed meat intake, 6 studies reported results^[22,23,26-28,43] but the association was positive and SS was found only in 2 studies^[22,28]. The largest study^[27] did not observe any association, but it was a study based on mortality cases with a relatively small number of food items included in the Food frequency questionnaires (FFQ). Salted, dried or preserved fish intake was associated (but not significantly) with GC risk only in one of the 5 cohort studies reporting results^[23-26,28], but in most of them the number of GC cases was too small. For pickled and dried vegetables, 3 studies found positive association but none of them achieved statistical significance^[23,25,26].

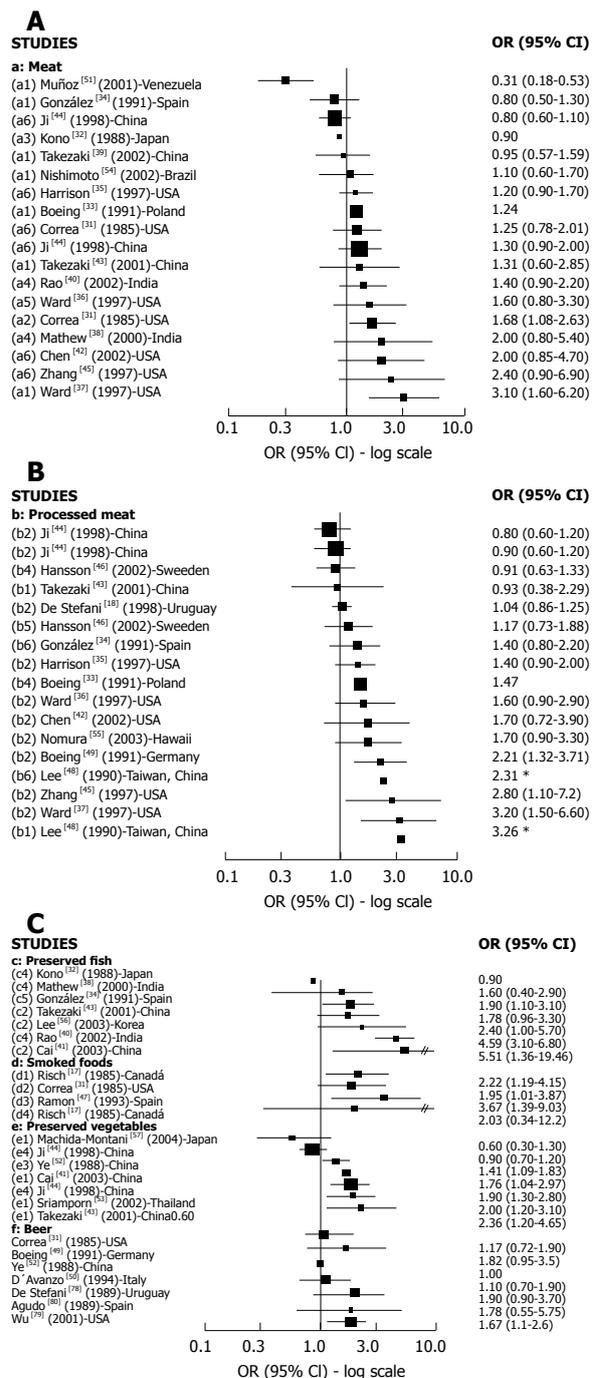


Figure 3 A: Meat intake and gastric cancer (case-control studies); (a) Meat intake: (a1) meat (a2) pork (a3) grilled meat (a4) mutton (a5) beef (a6) red meat; B: Processed meat and gastric cancer (case-control studies); * Without confidence intervals, but statistically significant; (b) Processed meat: (b1) salted meat (b2) processed meat (b3) bacon (b4) sausage (b5) cold cuts (b6) cured meat; C: Preserved fish, smoked foods, preserved vegetables and beer consumption and Gastric cancer (case-control studies); (c) Preserved fish: (c1) salted/dried fish (c2) salted fish (c3) processed fish (c4) dried fish (c5) preserved fish (d) Smoked foods: (d1) smoked meat (d2) smoked foods (d3) smoked/pickled (d4) smoked fish (e) Preserved vegetables: (e1) pickled vegetables (e2) dried vegetables (e3) salted vegetables (e4) preserved vegetables (f) Beer.

In the largest study^[26] the risk was borderline significant, but in the others the number of cases was too small. In relation to OC, (Figure 4A) we found 2 cohort studies^[29,30] reporting results associated with meat intake, which were positive and SS in one study^[29], while no association was observed regarding pickled vegetables.

Cases-control studies: We found 16 case-control studies

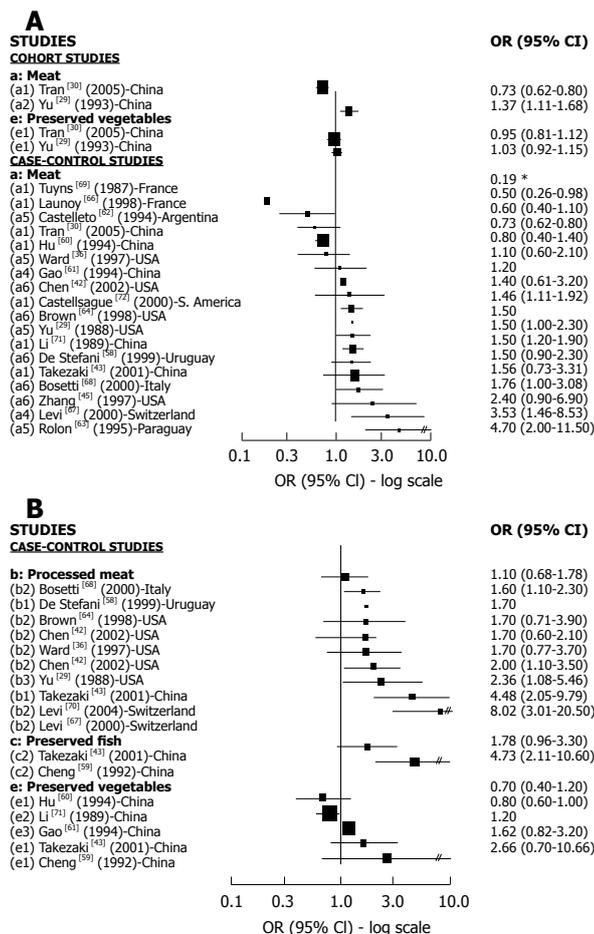


Figure 4 A: Meat and preserved vegetables and oesophageal cancer; * Without confidence intervals, but statistically significant; (a) Meat intake: (a1) meat (a2) pork (a3) grilled meat (a4) mutton (a5) beef (a6) read meat (e) Preserved vegetables: (e1) pickled vegetables (e2) dried vegetables (e3) salted vegetables (e4) preserved vegetables; **B:** Processed meat, preserved fish and preserved vegetables and oesophageal cancer (case-control and cohort studies); (b) Processed meat: (b1) salted meat (b2) processed meat (b3) bacon (c) Preserved fish: (e) Preserved vegetables: (e1) pickled vegetables (e2) dried vegetables (e3) salted vegetables.

(Figure 3A) reporting results between different types of meat intake (mutton, red meat, beef, fresh meat, grilled meat, pork) and GC risk^[31-40,42-45,51,54] and 11^[31,33,35-38,40,42-45] of them suggested a positive association with at least one type of meat intake and GC risk, which was statistically significant in 2 studies^[31,37] carried out in the USA. We found 14 publications^[18,33-37,42-46,48,49,55] with reported results on processed meat intake and GC risk (Figure 3B), and 12 of them^[18,33-37,42,45,46,48,49,55] showed a positive association while 4 were statistically significant^[37,45,48,49]. For dried/salted or preserved fish (Figure 3C), we found 7 studies^[32,34,38,40,41,43,56] reporting results, and 6 described a positive association with GC^[34,38,40,41,43,56] while 4 achieved statistical significance^[34,40,41,56]. Three studies published results about smoked food intake and GC risk^[17,31,65] and all of them showed a positive and significant association. For pickled and preserved vegetables, 5 of 6 studies^[41,43,44,52,53,57] showed a positive association with GC, which was statistically significant in all of them^[41,43,44,52,53]. Other food sources, such as beer^[31,49,50,52,78-80] were analyzed, but only one study showed a significant association with GC^[79].

Table 1 Overall quantification of epidemiological studies on nitrosamines and related food intake and gastric and oesophageal cancer risk

	Gastric cancer		Oesophageal cancer	
	<i>n</i> Studies (<i>n</i> positive association) [<i>n</i> SS]	<i>n</i> Studies (<i>n</i> positive association) [<i>n</i> SS]	<i>n</i> Studies (<i>n</i> positive association) [<i>n</i> SS]	<i>n</i> Studies (<i>n</i> positive association) [<i>n</i> SS]
Dietary exposure	Case-control	Cohort	Case-control	Cohort
Nitrites	7 (5) [3]	2 (1) [0]	2 (1) [0]	
Nitrosamines	5 (4) [3]	1 (0) [0]	1 (1) [0]	
Meat	16 (11) [2]	3 (2) [1]	18 (11) [6]	2 (1) [1]
Processed meat	14 (10) [4]	6 (2) [2]	9 (8) [5]	
Preserved fish	7 (6) [4]	5 (1) [0]	2 (2) [1]	
Preserved vegetables	6 (5) [5]	3 (3) [0]	5 (2) [0]	2 (0)
Smoked foods	3 (3) [3]			
Beer	7 (6) [1]			

SS: Statistically significant.

We found 18 case-control studies^[29,30,36,42,43,45,58,60-64,68,71,72] which published results on OC and different types of meat intake (Figure 4A). A positive association was observed in 11 of them^[29,42,43,45,58,63,64,66,67,70,71] and was SS in 6^[29,63,66,67,70,71]. For processed meat (Figure 4B), 8^[29,36,42,43,58,64,66,69] of 9 studies^[29,36,42,43,58,64,66,67,69] described a positive association with OC, which was SS in 5 studies^[29,43,58,67,69]. Only 2 studies^[43,59] publishing results on preserved fish intake and OC showed a positive association, being SS in one^[59]. The association between preserved vegetable intake and OC was reported in 5 studies^[43,59-61,70] and a positive but not significant association was observed in three of them^[43,59,61]. The two largest studies^[61,70] did not find any association.

DISCUSSION

Nitrite and nitrosamine intake

Although the evidence on nitrite and nitrosamine intake from cohort studies is limited (Table 1), results from case-control studies are quite consistent and support a positive relationship with GC. We did not find any results about the relation with OC from cohort studies and few case-control studies reported results, therefore a conclusion about the relationship between OC and nitrite and nitrosamine intake is impossible.

Meat intake

Only few cohort studies have reported results regarding the relationship between red meat intake and GC and OC, showing a positive association in 2 of 3 studies of GC and in 1 of 2 studies on OC. However, there are a large number of case-control studies presenting results on meat intake and GC and OC. Most of them have found a positive association (11 from 16 for GC and 11 from 18 for OC) particularly for OC, and in most of these studies the association is SS. Overall the evidence from case-control studies supports a positive association between meat intake and GC and OC. However, meat is a common substrate to endogenous formation of NOC and also a source of other carcinogenic compounds,

such as heterocyclic amines (HA) and polycyclic aromatic hydrocarbons (PAH) which should be taken into account.

Processed meat intake

Most of the relatively few cohort studies showing results on processed meat intake and GC risk have found no association between them. However, several of these studies considered only a small number of GC cases and food items. No cohort study has shown results on processed meat intake and OC. However a relatively large number of case-control studies have shown quite consistent results supporting a positive association between processed meat intake and GC and OC risk (10 of 14 studies on GC and 8 of 9 studies of OC), which were SS in most of them, particularly regarding OC.

Preserved fish intake

Regarding GC risk and preserved fish intake (particularly dried and salted), inconsistent results were found between case-control and cohort studies. While the case-control studies supported a positive association, the cohort studies did not, although most of them had a small number of cancer cases, and confidence intervals were wide. Therefore, further evidence is needed. No evidence is available from cohort studies in relation with OC and the few case-control studies showed a possible positive association.

Preserved vegetable intake

Results from case-control studies support a positive association between pickle and other preserved vegetable intake and GC risk. Almost all the studies have shown a positive and significant association. Cohort studies have also observed a positive but not significant association, although the number of cancer cases was small. Most of the studies were carried-out in Asian countries. To date, results on OC risk are inconsistent, but the number of studies is small.

Smoked food intake

Although the evidence is too limited for a definitive conclusion, it supports a positive association between smoked food intake and GC risk. No evidence exists for OC risk.

Beer drinking

So far the evidence is limited, but the majority of studies support a not significantly positive association with GC.

Limitation of evidence

Only 2 cohort^[11,12] and 9 case-control studies^[16,21,22-28] of 65 studies included in this review have published results on nitrite or nitrosamine intake in relation to GC or OC risk. This could be due to the absence of a complete food composition table for NOC content in foods^[72]. For the remainder, the estimation of dietary exposure to NOC and their precursors were done indirectly through foods identified as sources of them.

All the results could be affected by measurement

errors in the dietary intake, a common limitation of epidemiological studies. FFQ do not usually collect detailed and complete information about preservation and processing methods of all potential food sources of nitrosamines. In addition, the small number of food items usually included in the FFQ and/or lack of portion size information does not permit accurate estimation of nitrosamine and total food intake. The observed range of total food items in the FFQ varied between 22 and 81 in the studies used. Therefore, it could be expected that not all the studies have achieved an accurate assessment of the intake of these compounds. However, despite the fact that some studies have estimated adequately the exogenous intake of nitrosamines, none of them had information about endogenous NOC. It was reported that endogenous synthesis could contribute to 45%-75% of the total human exposure^[3]. However, recent studies carried out in humans have shown that endogenous NOC could be up 30-fold higher than exposure from dietary sources^[73-75], suggesting that we are actually measuring a small part of the total dietary human exposure to NOC, and therefore underestimating their effect.

In relation to possible factors that could modify the effect of NOC, few studies considered the intake of vitamin C or smoking habits. None of the studies on GC adjusted their results to consider *H pylori* infection. This is important because *H pylori* decreases the levels of vitamin C, a recognized inhibitor of endogenous nitrosamine formation^[76]. On the other hand, red meat is a source of iron which is considered an essential growth factor for *H pylori*^[77]. Therefore, some interaction with meat is expected. Finally, it is also important to take into account interactions with genes, particularly with polymorphisms of metabolic genes involved in the metabolism of NOC or DNA repair genes, which so far have been poorly studied.

Conclusions and future directions

At present, available epidemiological evidence from case-control studies on nitrite and nitrosamine intake supports a positive association with GC risk. The evidence in relation with OC is insufficient. There is quite consistent evidence from case-control studies about the positive association between meat and processed meat intake with both GC and OC risk. There is also quite consistent evidence from case-control studies about the positive association between preserved fish and preserved vegetable intake and GC risk, although results are more inconsistent in cohort studies. We have found a suggestive indication of a positive association between GC risk and smoked food intake. However, evidence about the effect of preserved fish and vegetable intake on OC risk is more limited, suggesting that there is no association between beer intake and GC although the evidence is still limited. Overall, more prospective cohort studies are needed to permit definitive conclusions, and should include a large number of cancer cases, dietary questionnaires with a large and detailed number of food items, good estimation of portion size, and control for all known confounding variables in a population with a wide range of food intake.

Evaluating the role of selected genotypes involved in

the metabolism of these chemical compounds and DNA repair potentially related to the risk of cancer, is also useful. On the other hand, taking into account that endogenous production seems to be the most important contributor to total NOC exposure, validated methodologies that allow an accurate assessment of production are needed. Therefore, measurement and quantification of DNA adducts of nitrosamines in humans may be the most direct way to assess both sources (exogenous and endogenous) and provide the best biomarker of exposure^[6].

In summary, prospective studies with long follow-up periods and validated methodologies quantifying all sources of exposure are needed to confirm the role of NOC in gastric and oesophageal carcinogenesis.

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COLORECTAL CANCER

E-cadherin expression pattern in primary colorectal carcinomas and their metastases reflects disease outcome

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E-cadherin can predict disease recurrence is obviously of great importance for both patients and clinicians, and significantly affects decisions concerning the therapy and management of the patients.

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Key words: Colorectal carcinoma; E-Cadherin membrane; Cytoplasmic immunohistochemistry; Prognosis; Disease-free survival; Disease-specific survival

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Abstract

AIM: To investigate the changes that occur in E-cadherin expression during the process of metastasis in colorectal cancer.

METHODS: E-cadherin expression was detected by immunohistochemistry and two indices of expression were calculated which reflected the level of expression and the locations (membrane and cytoplasm). Univariate and multivariate survival analyses were used to assess the value of these two E-cadherin indices as predictors of both disease-free (DFS) and disease-specific (DSS) survival.

RESULTS: E-cadherin membrane index (MI), but not cytoplasmic index (CI), was significantly higher in primary tumors than their metastases ($P = 0.0001$). Furthermore, both primary tumor MI and CI were higher among the patients who developed subsequent metastasis ($P = 0.022$ and $P = 0.007$, respectively). Interestingly, both indices were higher in liver metastase compared to other anatomic sites (MI, $P = 0.034$ and CI, $P = 0.022$). The CI of the primary tumors was a significant predictor of DFS ($P = 0.042$, univariate analysis), with a strong inverse correlation between CI and DFS ($P = 0.006$, multivariate analysis). Finally, the MI of primary tumor proved to be a significant independent predictor of DSS, with higher indices being associated with a more favorable outcome ($P = 0.016$).

CONCLUSION: Examination of E-cadherin expression and distribution in colorectal tumors can be extremely valuable in predicting disease recurrence. The observation that aberrant cytoplasmic expression of

INTRODUCTION

Colorectal cancer (CRC) is the second leading cause of cancer-related mortality in industrialized Western countries. The prognosis of CRC patients dramatically depends on the development of recurrence and/or metastasis. However, one of the major problems is the definition of reliable criteria for predicting recurrence and identifying the tumors that will respond to chemotherapy^[1,2].

The metastatic cascade starts with a breakdown of the epithelial integrity, which enables tumor cells to invade the surrounding stroma, penetrate either into blood or lymphatic vessels, and finally infiltrate into the appropriate target organs. Epithelial differentiation is critically dependent on maintenance of intact intercellular junctions by cell-cell adhesion molecules. Impairment of these junctions facilitates the invasion of epithelial cells, thus favoring the progression of carcinomas^[3]. There are several cell-cell adhesion molecules, including cadherins (E-, P-, N-cadherins), catenins (α , β , and γ catenins), and the CD44 family (standard and variants)^[4-6].

Of these cell-cell adhesion molecules, E-cadherin is normally located at the zonula adherens and is functionally necessary for epithelial integrity^[7,8]. Decreased levels of E-cadherin expression were reported in many immunohistochemical studies on epithelial malignancies^[9-11]. In some tumor types, including CRC, the loss of E-cadherin expression is associated with the loss

of tumor differentiation and is shown to be correlated with an increased likelihood of distant metastasis. These data suggest an important role of E-cadherin in tumor invasion and/or slowing down metastases^[12-14].

In the present study, we hope to extend these studies using immunohistochemistry to examine the levels of E-cadherin expression in primary tumors and their metastases from a series of 42 CRC patients. E-cadherin expression on the cell membrane and its cytoplasmic accumulation were assessed separately. Univariate and multivariate survival analyses were used to determine the potential predictive and prognostic value of E-cadherin expression in these patients.

MATERIALS AND METHODS

Patients, treatment and follow-up

A series of 42 patients were diagnosed and treated for Duke's B, C and D CRC at the Department of Oncology and Radiotherapy, Turku University Central Hospital and six other hospitals of the same hospital district between January 1996 and August 2003. The key clinical characteristics of the patients are summarized in Table 1. The patients were prospectively followed up until death or when they were seen alive for the last time, with a mean follow-up time of 32.3 mo (median 28.2) for the whole series in this study. At diagnosis, 30 patients had metastasis and the remaining patients developed metastatic diseases during follow-up. Disease-specific survival (DSS) and disease-free survival (DFS) were calculated based on the time from diagnosis to death (due to disease), and on the time from diagnosis to the appearance of metastatic diseases, respectively. In calculating the DSS, the patients who died from other or unknown causes were treated as censored cases.

Immunohistochemistry

Formalin-fixed, paraffin-embedded samples were available from all 42 primary tumors and their metastases. Paraffin blocks were cut on polylysine-coated microscopy slides for immunohistochemistry. The sections were deparaffinized in xylene, thrice for 10 min, then dehydrated in a descending series of ethanol (100%, 96%, 70%), followed by washes in TBS (0.05 mmol/L Tris-buffer physiological saline, pH 7.4-7.6), thrice for 5 min. Antigen retrieval was achieved by heating the samples without boiling in 10 mmol/L sodium citrate buffer, pH 6.0 (200 mL) in a microwave oven. This treatment was conducted twice for 7 min. The sections were washed in TBS buffer for 30 min. The endogenous peroxidase was blocked by 0.3% hydrogen peroxide in methanol for 20 min. The sections were washed in TBS for 15 min.

To inhibit non-specific binding, the samples were incubated in diluted normal horse serum (15 µL serum per 1 mL of TBS-buffer), in humid boxes for 30 min. The primary antibody was monoclonal mouse E-cadherin antibody (clone HECD-1, sub-class IgG₁, Zymed Laboratories, San Francisco, CA, USA), at a dilution of 1:300 from the stock. The dilution was based on dilution experiments. Bound primary antibody was visualized with

Table 1 Characteristics of patients and their tumors

Variables	n (%)
Patients	42 (100)
Female	25 (59.5)
Male	17 (40.5)
Primary tumor status (T)	
T1	0 (0.0)
T2	1 (2.4)
T3	35 (83.3)
T4	6 (14.3)
Primary nodal status (N)	
N0	19 (47.5)
N1	21 (52.5)
Duke's staging	
B	9 (21.4)
C	9 (21.4)
D	24 (57.1)
Histological grade	
I	9 (21.4)
II	27 (64.3)
III	6 (14.3)
Site of metastasis	
Local	6 (14.3)
Liver	13 (31.0)
Ovary	3 (7.1)
Peritoneum	3 (7.1)
Mesentery	4 (9.5)
Omentum	6 (14.3)
Lymph node	2 (4.8)
Other ¹	5 (11.9)

¹Lymph node, perineum, urinary bladder, lung and brain.

the avidin-biotin peroxidase technique (Vectastain ABC kit, Vector Laboratories, Burlingame, CA, USA). The antibody was diluted with 20 mmol/L TBS, pH 7.4 (10 mmol/L CaCl₂, 0.1% NaN₃ and 1% BSA). The sections were incubated in the diluted antibody and the negative controls in 1% BSA in TBS-buffer. The antibody and the 1% BSA incubations were done with 195 µL of the incubation fluid, which was enough to cover the whole section. The incubation took place in incubation boxes at 4°C overnight. After the incubation, the boxes containing sections were left at room temperature for 30 min. The incubation fluid (primary antibody or bovine albumin) was washed off with TBS-buffer (two washes). The secondary antibody (4.5 µL biotinylated anti-mouse antibody in 1 mL of 1% BSA) was pipetted onto the sections and incubated in the moist box for 30 min. The secondary antibody was washed in TBS buffer for 15 min. Vectastain ABC reagent was then used to stain the fixed secondary antibody. After 30 min of incubation, the sections were washed with TBS-buffer.

The final staining was done in diaminobenzidine tetrahydrochloride (DAB) solution (49 mL TBS-buffer, 34 mg imidazole, 17 µL 30% hydrogen peroxide and 1 mL 30% DAB), for 5 min. The slides were washed with distilled water, 70% ethanol for 1 min, then in distilled water. The nuclei were stained with Mayer's hematoxylin

for 30 s. Extra stain was washed with tap water. The slides were then transferred through ascending ethanol series, and xylene before mounting.

Evaluation of E-cadherin staining

The E-cadherin staining was evaluated by an observer blinded to the clinical data (AE) under regular light microscopy. Only the infiltrating part of the neoplasm was evaluated. Both the membranous and cytoplasmic staining were evaluated separately. For cell membrane staining, four categories were used (+++, ++, +, -), starting from equivalent to normal through to entirely negative^[10]. The cytoplasmic staining was also graded into four categories: (0) negative, no detectable staining, (1) weak, but still detectable staining, (2) moderate, clearly positive but still weak, and (3) heavy staining, intense. In calculating the staining index; membrane (MI) and cytoplasmic (CI), both the intensity of staining and the fraction of positively stained cells were taken into account, using the following formula:

$$I = 0*f_0 + 1*f_1 + 2*f_2 + 3*f_3$$

where, I is the staining index, f_0 - f_3 the fractions of the cells showing a defined level of staining intensity (from 0 to 3). Theoretically, the index could vary between 0 and 3^[15]. The reproducibility of the E-cadherin staining indices was tested twice by one of the observers (AE) analyzing the sections, after a few days (intra-observer variation).

Statistical analysis

Statistical analyses were made using SPSS® (SPSS, Inc., Chicago, USA) and STATA (Stata Corp., TX, USA) software packages (SPSS for Windows, version 12.0.1 and STATA/SE 8.2). Frequency tables were analyzed using the χ^2 test, and likelihood ratio (LR) or Fisher's exact test was used to assess the significance of the correlation between the categorical variables. Odds ratios and their 95% confidence intervals (95%CI) were calculated where appropriate by the exact method. Differences in the means of continuous variables were analyzed using non-parametric tests (Mann-Whitney or Kruskal-Wallis) for 2- and K-independent samples, respectively. Analysis of variance (ANOVA) was only used for deriving the mean values and their SD of each individual category. Bivariate correlation (Spearman rho) and scatter plots were used to check the correlations between the two continuous variables (MI, CI vs DFS, DSS), controlled by linear regression analysis (R and R^2) for linearity. Univariate survival (life-table) analysis for the outcome measure (DSS, DFS) was based on Cox's method (indices treated as continuous variables). Multivariate survival analysis was made using Cox's proportional hazards model in a backward stepwise manner with the log-likelihood ratio (L-R) significance test, and using the default values for enter and exclusion criteria. The assumption of proportional hazards was controlled by log-minus-log (LML) survival plots. In all tests, the values $P < 0.05$ were regarded statistically significant.

RESULTS

E-cadherin expression was assessed in all tumors (Figure 1).

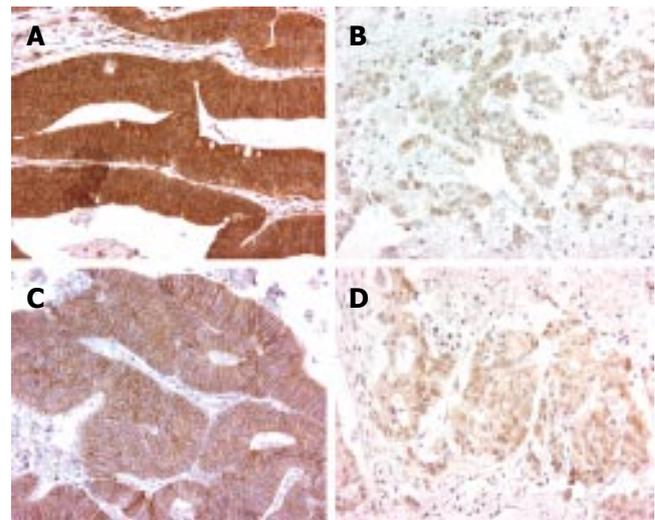


Figure 1 Immunohistochemical staining patterns. **A:** Primary tumor showing high E-cadherin membranous and cytoplasmic indices (MI = 1.75, CI = 0.85); **B:** Metastatic disease showing low E-cadherin indices (MI = 0.40, CI = 0.25); **C:** Metastatic disease in the liver showing high E-cadherin indices (MI = 1.00, CI = 0.60); **D:** Metastatic disease in the colon showing low E-cadherin indices (MI = 0.35, CI = 0.25).

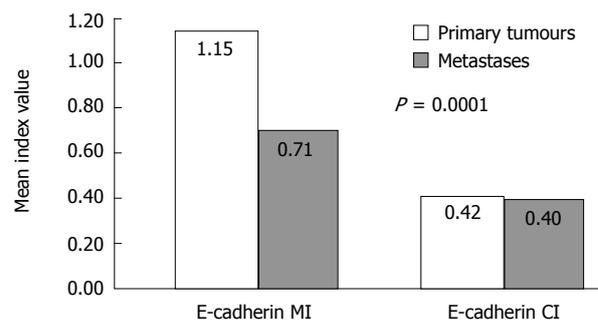


Figure 2 Comparison of E-cadherin indices in primary tumors and metastases.

The expression pattern of E-cadherin was predominantly membranous in normal colonic epithelium but this pattern was disturbed (diffuse cytoplasmic and membranous) in the tumor area.

E-cadherin indices in primary tumors and metastases are shown in Figure 2. Interestingly, MI was significantly higher in primary tumors compared with their metastases ($P = 0.0001$), whereas CI did not show such difference. Both membrane and cytoplasmic E-cadherin expression in the primary tumor was increased in patients who developed metastases ($P = 0.022$ and $P = 0.007$, respectively).

Among the metastatic lesions, MI and CI were related to the site of metastases as shown in Figure 3, both indices were higher in metastases of the liver than in those at other anatomic sites ($P = 0.034$ and $P = 0.022$, respectively).

E-cadherin CIs were significantly higher in the primary tumors from patients who were above the median age of 67.5 years compared with those who were below ($P = 0.044$). MI and CI did not significantly correlate with other clinical variables such as sex, tumor grade, tumor stage, etc.

The Cox model was used to analyze the power of E-cadherin indices as predictors of DFS and DSS, using univariate mode for each of the values separately, followed

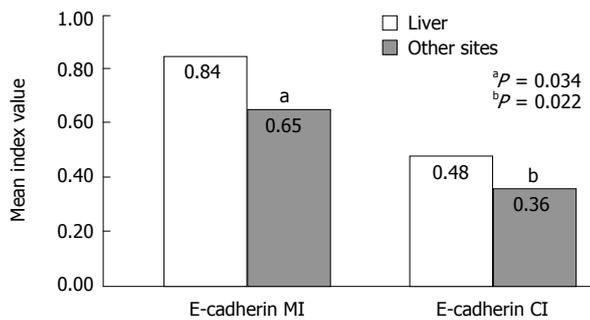


Figure 3 Comparison of E-cadherin indices and site of metastases.

by the multivariate test of all index values entered in the model. In univariate analysis, only the CI in the primary tumors was a significant predictor of DFS, with OR of 3.25 (95%CI 1.04-10.13) ($P = 0.042$). When all indices were entered in the multivariate Cox model, the CI of the primary tumor retained its value as an independent predictor of DFS, with OR of 4.65 (95%CI 1.36-15.89), while the others dropped out from the model. Indeed, there was a strong inverse correlation between CI and DFS, as shown in the scatter plot (Figure 4), cytoplasmic expression in the primary tumor was significantly higher in patients with disease recurrence ($n = 26$) during the follow-up period than those without (R^2 linear = -0.126, and $r = 0.354$ in linear regression analysis, as well as in bivariate correlation) (Spearman rho = -0.418; $P = 0.006$). This independent predictive power of the CI was not confounded by lymph node status, tumor location, primary T, grade, or age (continuous or dichotomized). In fact, predictive power increased in the final model to OR 7.30 (95%CI 1.51-35.10) ($P = 0.013$).

In the analysis for DSS, none of the indices in either the primary tumor or the metastasis were significant predictors of DSS in the univariate mode. However, in the multivariate model, adjusted for all recorded variables, MI in the primary tumor proved to be a significant independent predictor of survival, in that the higher indices were associated with a more favorable outcome, OR 0.332 (95%CI 0.136-0.813) ($P = 0.016$). In scatter plot, R^2 linear was 0.058 and in linear regression, $r = 0.240$ (Spearman rho = 0.267, $P = 0.091$). None of the other variables in the model proved to be significant independent predictors of DSS.

DISCUSSION

Cancer metastasis occurs through various steps and many biological factors are involved in these processes^[16]. Among these, the adhesion molecules are closely involved in the development and growth of metastatic tumors^[17]. If the activity of adhesion molecules is decreased, cells tend to detach from their site of origin. Conversely, the reattachment of tumor cells at the sites of metastases is mediated by multiple adhesion molecules, including integrins, selectins and cadherins^[18].

Colorectal epithelial cells of the crypt normally exhibit strong membranous expression of E-cadherin at cell-cell borders. This reflects the normal localization of this

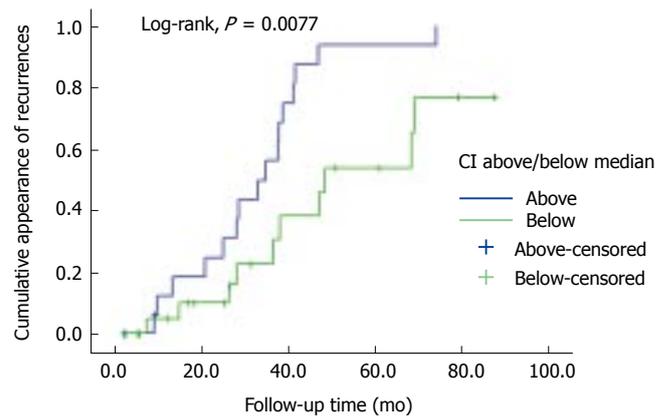


Figure 4 Association of recurrence with E-cadherin CI indices in metastases.

intercellular adhesion molecule^[19]. When we assessed the level and pattern of expression of E-cadherin in primary colorectal tumors and their metastases, membranous and cytoplasmic expression at both locations was consistent with the previous reports^[20-23]. Multiple mechanisms are known to cause abnormal E-cadherin expression, including mutations or deletions of the E-cadherin gene itself, mutations in the β -catenin gene, and transcriptional repression of the E-cadherin gene, e.g., by hypermethylation or chromatin rearrangements in the E-cadherin promoter region^[24,25]. It is not yet clear which of these are relevant in CRC. However, in this study, the presence of both membranous and cytoplasmic expression in some tumors suggests that the E-cadherin molecule is still capable of locating the membrane, but its abnormal presence in the cytoplasm may be due to other reasons.

In this study, primary tumors showed significantly higher membranous expression of E-cadherin compared with the metastases, while cytoplasmic expression did not differ between the two tumor groups. This may reflect the selection of cells with less E-cadherin expression as metastases are formed, or possibly the local environment of the organ in which the metastasis has been formed. Our findings suggest that the site of metastasis is associated with the distribution of E-cadherin expression. There were differences in E-cadherin expression (both CI and MI) among the metastatic lesions at different anatomic locations. Both MI and CI were significantly higher in metastatic lesions in the liver than in metastasis at other sites (Figure 3). Although these observations implicate several possible mechanisms, there is little data to draw any definitive conclusions; however, Bongiorno *et al* reported that cancer cells in thoracic neoplasms and in Barrett's esophagus lost their E-cadherin expression transiently during their dissemination, and then re-expressed this and other adhesion molecules at a distant metastatic site^[26]. This concept is indirectly supported by the present observations that MI was significantly higher in primary tumors than in their metastases, whereas CI did not show such a difference. Our findings justify the need to examine the expression of other adhesion molecules so as to investigate the possibility that circulating cancer cells with a distinct adhesion molecule pattern would be preferentially selected to deposit in the liver. Some debate

exists concerning the changes in the level of E-cadherin expression in the primary CRC and its metastasis. Gagliardi and co-workers compared 14 liver metastases with their corresponding primary tumors and found complete loss of membranous E-cadherin expression in 7/14 (50%) liver metastases of which 6/7 (86%) showed intense expression in the corresponding primary tumors^[19], while Ikeguchi and colleagues reported increased E-cadherin expression in up to 40% of the liver metastases compared with only 17% of the metastatic lymph nodes that were studied^[27]. Similarly, Mayer *et al* reported that in many gastric carcinomas, E-cadherin expression in metastatic lesions of the liver was indeed stronger than that in the primary tumors^[28].

When we looked at the patients with metastases at the time of diagnosis ($n = 30$), we found that CI was almost double in their primary tumors, suggesting that increased aberrant distribution of E-cadherin in the primary tumors may reflect an increased likelihood of metastasis formation. Furthermore, cytoplasmic expression in the primary tumors was significantly higher in patients with disease recurrence ($n = 26$) during the follow-up period than those without recurrence. The E-cadherin MI was very similar among those who had metastasis at onset and in those who developed recurrence during follow-up (1.13 *vs* 1.25, respectively). In addition, CI values in the primary tumors of patients who had primary metastasis (0.48) were very similar to those who had subsequent recurrence (0.54). In both cases, CI was always lower among those without primary metastasis and in those who did not develop recurrent disease, (0.27 and 0.22, respectively). These findings suggest that cytoplasmic (aberrant) E-cadherin expression more closely reflects the adverse biological behavior of CRC than does membranous (normal) expression.

In univariate analysis, the cytoplasmic E-cadherin index in primary tumors was a significant predictor of DFS, and CI retained its independent predictive power of DFS also in the multivariate model. In fact, its predictive power substantially increased in the final multivariate model. This effect is explained by the strong inverse correlation between CI levels and DFS (Figure 4). The CI did not predict DSS, however, in either univariate or multivariate model. Interestingly, the MI of the primary tumor proved to be a significant predictive factor in multivariate analysis (with OR and 95%CI far below 1.0), indicating that higher MI values are associated with more favorable disease outcome, suggesting that membranous (normal) E-cadherin expression may reflect a tumor that retains a more normal cell phenotype.

Certainly, our findings support the concept that aberrant (cytoplasmic) E-cadherin expression is a sign of adverse disease outcome in CRC. These results are consistent with the previous reports that loss of normal expression of E-cadherin is associated with poor prognosis in breast and colorectal carcinoma^[29,30]. The present study further confirms the power of E-cadherin indices as predictors of disease recurrence and progression.

This study found no significant correlation between E-cadherin indices and the various clinical variables recorded (sex, tumor grade, tumor stage, *etc*); however, this

is not surprising since other groups have also reported similar findings^[31].

Finally, the importance of distinguishing between membrane and cytoplasmic E-cadherin expression is emphasized by this study, because it seems to reflect different aspects of the behavior of CRC. Cytoplasmic expression more closely predicts disease recurrence and aberrant expression (downregulation) at the membrane seems to indicate a poorer prognosis. In both cellular locations, E-cadherin expression is reduced in metastasis as compared with the primary tumors. We can only speculate at the mechanisms of this change in expression, and it may be due to alterations in catenins, which link the cadherin molecules to the actin cytoskeleton. Similarly, the observed higher levels of E-cadherin expression (both MI and CI) in metastatic deposits in the liver than in other sites need further investigations.

To conclude, it is clear that examining E-cadherin expression and distribution in colorectal tumors is extremely valuable in predicting disease recurrence. The observation that aberrant cytoplasmic expression of E-cadherin can predict disease recurrence is obviously of great importance for both the patient and the clinician, and affects significantly decisions concerning the therapy and management of the patients.

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VIRAL HEPATITIS

Mechanism of T cell hyporesponsiveness to HBcAg is associated with regulatory T cells in chronic hepatitis B

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CONCLUSION: The results indicate that the mechanism of T cell hyporesponsiveness to HBcAg includes activation of HBcAg-induced regulatory T cells in contrast to an increase in T_H2-committed cells in response to HBsAg.

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Key words: Hepatitis B virus; Regulatory T cells; IL-10; FOXP3; T_H1

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Abstract

AIM: To study the mechanisms of hyporesponsiveness of HBV-specific CD4⁺ T cells by testing T_H1 and T_H2 commitment and regulatory T cells.

METHODS: Nine patients with chronic hepatitis B were enrolled. Peripheral blood mononuclear cells were stimulated with HBcAg or HBsAg to evaluate their potential to commit to T_H1 and T_H2 differentiation. HBcAg-specific activity of regulatory T cells was evaluated by staining with antibodies to CD4, CD25, CTLA-4 and interleukin-10. The role of regulatory T cells was further assessed by treatment with anti-interleukin-10 antibody and depletion of CD4⁺CD25⁺ cells.

RESULTS: Level of mRNAs for T-bet, IL-12R β2 and IL-4 was significantly lower in the patients than in healthy subjects with HBcAg stimulation. Although populations of CD4⁺CD25^{high}CTLA-4⁺ T cells were not different between the patients and healthy subjects, IL-10 secreting cells were found in CD4⁺ cells and CD4⁺CD25⁺ cells in the patients in response to HBcAg, and they were not found in cells which were stimulated with HBsAg. Addition of anti-IL-10 antibody recovered the amount of HBcAg-specific T_H1 antibody compared with control antibody ($P < 0.01$, 0.34% ± 0.12% vs 0.15% ± 0.04%). Deletion of CD4⁺CD25⁺ T cells increased the amount of HBcAg-specific T_H1 antibody when compared with lymphocytes reconstituted using regulatory T cells ($P < 0.01$, 0.03% ± 0.02% vs 0.18% ± 0.05%).

INTRODUCTION

Hepatitis B virus (HBV) is a noncytotoxic DNA virus which causes chronic hepatitis and hepatocellular carcinoma as well as acute hepatitis^[1]. HBV now affects more than 300 million people worldwide^[2] and in approximately 5% of adults and 95% of neonates who become infected with HBV, persistent infection develops.

It has been shown that cytotoxic T lymphocytes (CTLs) play a central role in the control of virus infection^[3]. In addition, CD4⁺ T cells provide help for both CTLs and B-cell responses^[4]. Hyporesponsiveness of HBV-specific T cells in peripheral blood has been shown in patients with chronic HBV infection^[5]. Recently, lamivudine treatment in chronic hepatitis B has been reported to restore both CD4⁺ T cells and CTL hyporesponsiveness following the decline of serum levels of HBV DNA and HBsAg^[6,7]. However, previous reports have indicated that HBV-specific T cells restored by lamivudine treatment are insufficient to completely suppress HBV replication^[8,9]. In our previous study, we observed a defect in recovery of HBcAg-specific T_H1 cells despite restoration of CTLs, although they showed limited functions^[10,11]. Since type 1 helper T (T_H1) cells are believed essential for immunity against intracellular pathogens^[12], more detailed study of HBV-specific CD4⁺ cells is needed in order to understand the mechanisms of persistent infection in CHB.

Increasing evidence has suggested that both cytokine

balance including interferon- γ (IFN- γ) and interleukin-4 (IL-4) and direct signaling through the T cell receptor is important for T_H1 and T_H2 commitment^[13]. The critical transcription factors for commitment of T cells to the T_H1 or T_H2 pathway are T-bet or GATA-3 respectively^[14-16]. Whether various antigens derived from the HBV genome affect expression of these factors is unknown. It is important to understand how cytokine balance and antigen types could affect T_H1/T_H2 commitment in chronic hepatitis B.

There have also been reports about the possible induction of anergy by regulatory T cells (T_{reg} cells), that constitutively express CD25 (the IL-2 receptor alpha-chain) in the physiological state^[17-19]. In humans, this T_{reg} population, as defined by CD4⁺CD25⁺CTLA-4⁺ expression, constitutes 5% to 10% of peripheral CD4⁺ T cells and has a broad repertoire that recognizes various self and nonself antigens. It has been indicated that T_{reg} cells have several different mechanisms for suppressing various kinds of immune cells^[20,21]. The important mechanisms are cell to cell contact and secretion of cytokines including IL-10 and transforming growth factor-beta (TGF-beta)^[22-26]. Antigens derived from HBV might induce T_{reg} cells to escape from immunological pressure as reported in persistent infection of EB virus, hepatitis C virus and HIV-1^[24,26,27].

In this study we examined the mechanisms of hyporesponsiveness of HBV-specific CD4⁺ T cells by evaluating the T_H1/T_H2 commitment and activity of T_{reg} cells.

MATERIALS AND METHODS

Study design

Nine patients with chronic hepatitis B (CHB) were enrolled in this study (Table 1). The patients had more than 5.0 log genome equivalent (LGE /mL; Chugai Pharmaceutical Co., Tokyo, Japan) of serum HBV DNA and had elevated alanine aminotransferase (ALT) values (normal range < 40 IU/L) for more than 6 mo prior to the study. Six patients were seropositive for HBeAg and three patients were seropositive for anti-HBe. All the patients were negative for antibodies to hepatitis C virus (HCV) and did not have liver diseases due to other causes, such as alcohol, drug, congestive heart failure and autoimmune disease. For control subjects, ten healthy HBsAg-vaccinated subjects were included.

Permission for the study was obtained from the Ethical committee at Tohoku University School of Medicine. Written informed consent was obtained from all the subjects enrolled in this study. The study comprised 6 mo of monitoring before obtaining peripheral blood with assessments at 1, 2, 4, and 6 mo. At each assessment, patients were evaluated for serum HBV DNA, HBeAg, anti-HBe, blood chemistry and hematology. HBsAg, anti-HBs, total and IgM anti-HBc, HBeAg, anti-HBe, and anti-HCV were determined by commercial enzyme immunoassay kits (Abbott Laboratories, Chicago, IL). Serum levels of HBV DNA were measured by transcription mediated amplification-hybridization protection assay (lower limit of detection: 3.7 LGE/mL).

Table 1 Summary of clinical characteristics of patients with chronic hepatitis B enrolled in the study

Case	Age (yr)	Gender	ALT (IU/L)	HBeAg (Cutoff index)	Anti-HBe (Inhibition %)	HBV DNA (LGE/mL)	HBV Genotype
1	55	M	78	67	< 0.5	5.8	C
2	36	M	183	100	< 0.5	7.6	ND
3	31	M	50	66.9	< 0.5	7.6	C
4	42	M	141	100	< 0.5	6.8	C
5	27	M	77	75.7	< 0.5	7.6	C
6	42	F	42	93.8	< 0.5	7.0	C
7	32	M	70	< 0.5	100	6.2	C
8	29	M	81	< 0.5	86.9	5.3	C
9	58	M	117	0.7	100	7.3	C

The values for serum levels of ALT, HBeAg, anti-HBe, HBV DNA and HBV genotypes were determined at the time of blood sampling. Abbreviations: M, male; F, female; LGE/mL, log genome equivalent /mL; ND, not determined.

Reagents

IL-10 and IFN- γ secretion assay kits were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). Monoclonal antibodies to human CTLA-4 (APC-labeled), CD4 (PerCP-labeled), CD3 (FITC-labeled), CD25 (FITC-labeled), IL-10 (No Azide / Low Endotoxin) and isotype-matched control antibodies were purchased from BD Biosciences Pharmingen (San Diego, CA). HBsAg and HBeAg were obtained from Biodesign International (Saco, MA).

Cell culture

Peripheral blood mononuclear cells (PBMCs) isolated from heparinized blood by Ficoll-Hypaque density gradient centrifugation were resuspended in RPMI 1640 supplemented with 8% human AB serum (Nabi, Miami, FL; complete medium) and were cultured in a 96-well plate at a concentration of 1×10^7 cells/mL in complete medium in the presence of HBsAg (29 μ g/mL) or HBeAg (10 μ g/mL) for 24 h. Thereafter, CD4⁺ cells (4×10^5 cells) were separated from the stimulated PBMCs using anti-CD4-coated magnetic beads (Dynabeads M-450 CD4, DYNAL, Oslo, Finland) for quantification of mRNAs.

Quantified real time PCR

Total cellular RNA was extracted from CD4⁺ cells using an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instruction. Contaminating small DNA was removed by DNase I digestion using an RNase-free DNase system (Qiagen). Subsequently, total RNA was reverse-transcribed to single strand cDNA using random hexamers. In brief, the amount of extracted RNA was measured by NanoDrop ND-1000 (NanoDrop Technologies, Rockland, DE). After mixing with random primers and DEPC water, 1 μ g RNA was further mixed with $5 \times$ first strand buffer, dNTP mixture and 0.1 mol/L DTT. After preincubation (25°C, 10 min), M-MLV RT (Takara, Tokyo, Japan) and ribonuclease inhibitor were added and samples were incubated further for 60-min at 37°C. Realtime PCR was performed on an ABI PRISM 7700 Sequence Detector (Perkin-Elmer Applied Biosystems, Foster City, CA) using predeveloped TaqMan Assay Reagents (Perkin-Elmer

Applied Biosystems) according to the manufacturer's protocol^[28]. The commercially available primers and probe for the amplification of T-bet (ID Hs00203436), IFN- γ (ID Hs00174143), GATA-3 (ID Hs00231122), IL-4 (ID Hs00174122), FOXP3 (ID Hs00203958) and GAPDH were purchased from Perkin-Elmer Applied Biosystems. Amplification of IL-12R β 2 was performed as previously described^[29].

IL-10 and IFN-gamma secretion assay

Purified PBMCs were stimulated at 1×10^7 cells/mL in complete medium with or without HBcAg (10 μ g/mL) for 9 h at 37°C. Cells were washed by adding 2 mL of cold buffer and resuspended in 90 μ L of cold medium. After the addition of 10 μ L of IL-10- or IFN-gamma-capture Reagent, cells were incubated for 5 min on ice. Thereafter, cells were diluted with 1 mL of warm medium (37°C) and further incubated in a closed tube for 45 min at 37°C under slow continuous rotation. Cells were washed and IL-10- or IFN- γ -secreting cells were stained by adding 10 μ L of IL-10- or IFN- γ -Detection Antibody (PE-conjugated) together with anti-CD4-PerCP and anti-CD25-FITC. In some experiments, FITC fluorescence was amplified by FASER kit-FITC (Miltenyi Biotec). Selected samples were stained with anti-CD14-FITC, anti-CD3-PerCP, anti-HLA-DR-APC (BD Biosciences). Cells were analyzed by FACSCalibur.

To assess the effects of IL-10 on the HBcAg-specific IFN- γ production by CD4⁺ T cells, PBMCs were stimulated at 1×10^7 cells/mL in complete medium with or without HBcAg (10 μ g/mL) and with or without anti-human IL-10 monoclonal antibody at the indicated concentration for 9 h at 37°C. Cells were then used for IFN- γ -secretion assay and analyzed by FACSCalibur.

Intracellular and surface CTLA-4 staining

In order to analyze the expression of total CTLA-4 in CD4⁺CD25⁺ cells, cells were fixed and permeabilized using BD cytofix/cytoperm solution (BD Bioscience) after cell surface markers including CTLA-4 were stained. Subsequently, intracellular CTLA-4 was stained and the cells were analyzed by FACSCalibur^[30].

Depletion of T_{reg} cells

By using the CD4⁺CD25⁺ Regulatory T Cell Isolation Kit (Miltenyi Biotec), three fractions of lymphocytes were obtained; lymphocytes depleted of CD4⁺ cells (fraction 1), purified CD4⁺CD25⁺ lymphocytes (fraction 2) and purified CD4⁺CD25⁺ cells (fraction 3). To test the effect of CD4⁺CD25⁺ cells on HBcAg-specific IFN- γ production, 2 sets of lymphocyte preparations were reconstituted. The first set, designated as T_{reg}⁺, was the mixture of all three fractions and contained 5%-7% CD4⁺CD25⁺ cells. The second set, designated as T_{reg}⁻, was the mixture of fractions 1 and 2, and contained 0.5% (mean) of CD4⁺CD25⁺ cells.

Statistical analysis

Differences in the amounts of cytokines produced were analyzed by oneway ANOVA between patients with CHB and healthy controls. The frequencies of cytokine-secreting cells were analyzed by Mann-Whitney *U* test.

Both tests were run using SPSS ver. 10. A level of $P < 0.05$ was considered as being statistically significant.

RESULTS

Expression of mRNA relating to T_H1/T_H2 commitment in CD4⁺ cells

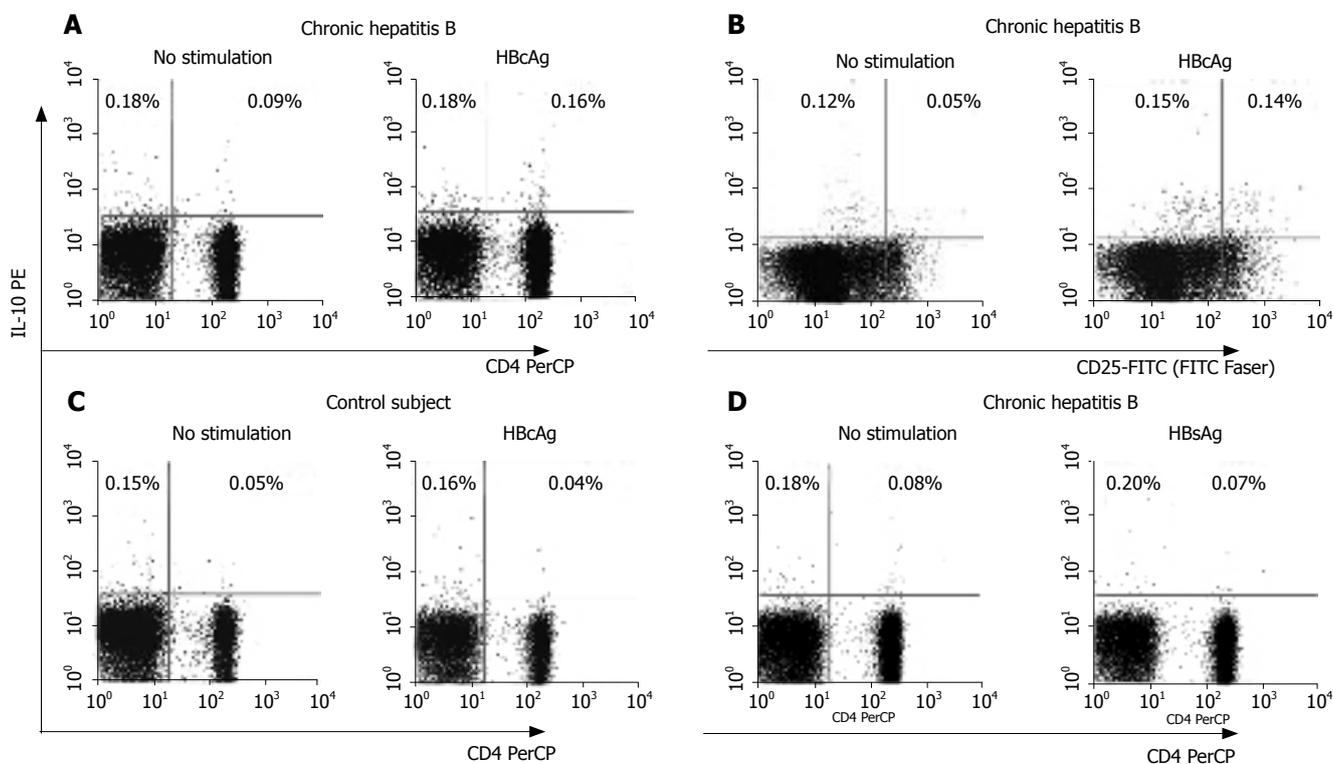
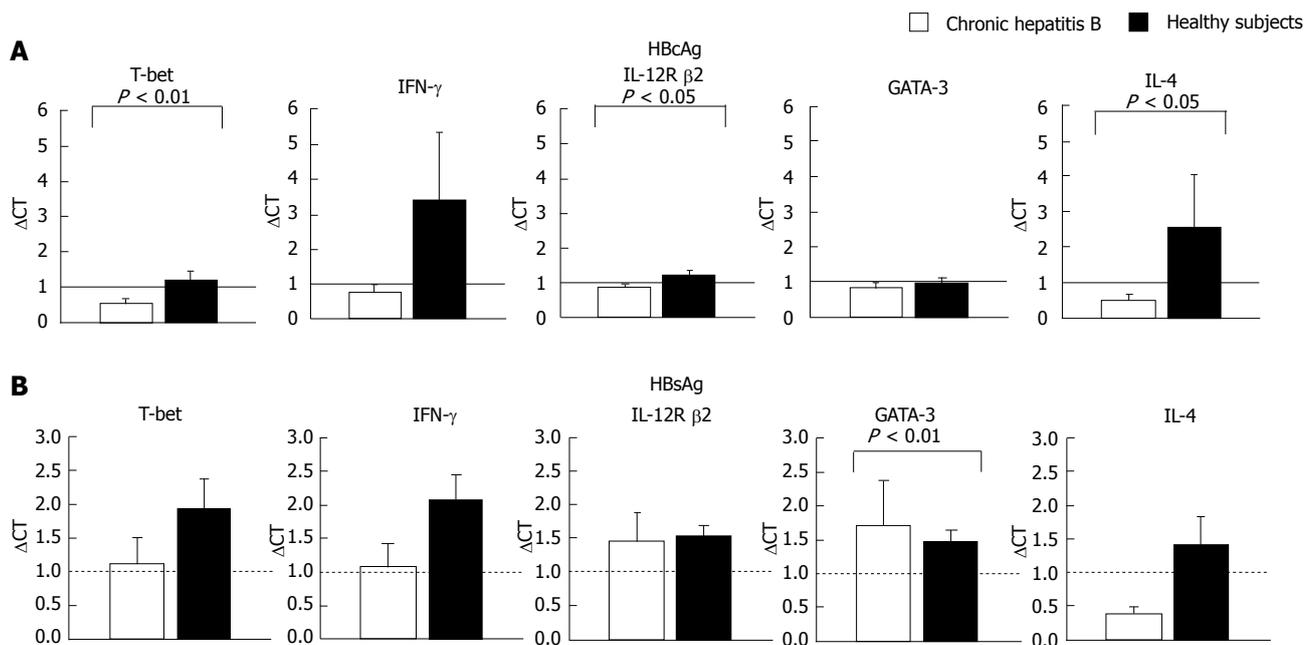
In CHB patients, HBcAg significantly suppressed the expression of mRNAs for T-bet ($P < 0.01$), IL-12R β 2 ($P < 0.05$) and IL-4 ($P < 0.05$) compared with those of healthy volunteers (Figure 1A). In addition, the expression levels of mRNAs for IFN- γ and GATA-3 were below 1.0 in response to HBcAg stimulation (Figure 1A). On the other hand, HBsAg induced the upregulation of GATA-3 mRNA compared with healthy volunteers ($P < 0.01$) while the expression level of T_H1 related mRNA (T-bet, IFN- γ , and IL-12R β 2) remained unchanged (Figure 1B).

IL-10 secreting cells in response to HBcAg were enriched in CD4⁺CD25⁺ lymphocytes

Involvement of the suppressive cytokine IL-10 in suppression of T_H1-commitment of HBcAg-stimulated cells was evaluated by enumeration of IL-10-secreting cells. Since the cells secreting IL-10 were mostly found in the CD3⁺ population, cells were further studied by staining with antibodies to CD4 and CD25. A population of IL-10-secreting CD4⁺ T cells was readily detectable in patients with CHB (Figure 2A) and these IL-10 secreting cells in CD4⁺ T cells showed CD25^{high} expression (Figure 2B), while there were no such responding cells in healthy subjects (Figure 2C). In addition, when the cells were stimulated with HBsAg, no IL-10 producing CD4⁺CD25^{high} cells were detected (Figure 2D). The percentage of HBcAg-specific IL-10 secreting CD4⁺ cells in all patients with CHB was $0.10\% \pm 0.04\%$ (mean \pm standard deviation), and the population was more prominent in CD4⁺CD25^{high} cells (Figure 3). Our next question was whether T_{reg} cells increased in number or were induced by HBcAg stimulation. Therefore, the population of CD4⁺CD25^{high}CTLA-4⁺ T cells was compared between CHB patients and healthy subjects (Figure 4A). However, no statistical difference in the population with this phenotype was found between normal subjects and CHB patients (Figure 4B).

Recovery of IFN- γ -secreting cells by the addition of anti-IL-10 antibody

Low response of HBcAg-specific T_H1 cells defined by IFN-gamma-secreting CD4⁺ T cells in response to HBcAg stimulation was indicated by the lack of statistical difference in that population between patients with CHB and normal subjects (Figure 5A). To further assess the role of IL-10 in the suppression of T_H1 responses to HBcAg stimulation, the effect of anti-IL-10 antibody on T_H1 response was evaluated by addition of anti-IL-10 cultures. The population of CD4⁺ T cells was comparable when cultured with and without anti-IL-10 antibody (Figure 5B). In the presence of anti-IL-10 antibody, the population of IFN- γ -secreting CD4⁺ T lymphocytes in response to HBcAg significantly increased (2.3-fold, $0.34\% \pm 0.12\%$; mean \pm SD of 9 cases) compared to culture with a control



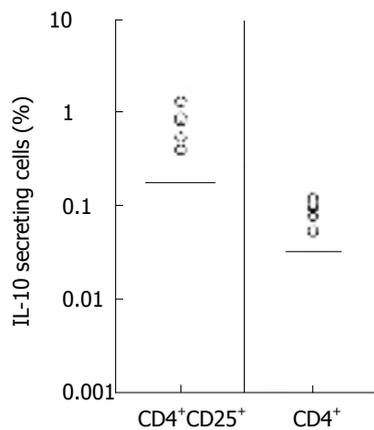


Figure 3 Increased populations of HBcAg-specific IL-10-producing CD4⁺ or CD4⁺CD25^{high} T cells in patients with chronic hepatitis B. Population of IL-10 secreting cells in CD4⁺ T cells and in CD4⁺CD25⁺ T cells was evaluated in patients with CHB. Frequencies of HBcAg-specific IL-10 secreting cells were calculated by subtracting percentage in non-stimulated samples from percentage in HBcAg-stimulated samples. Upper limits of normal subjects (mean \pm 2SD of 5 subjects) were shown by straight lines in the plots (0.14% for CD4⁺CD25⁺ cells and 0.027% for CD4⁺ cells). A FITC faser kit (BD Bioscience Pharmingen) was used in some experiments of ease separation of positive events by enhancing fluorescence intensity.

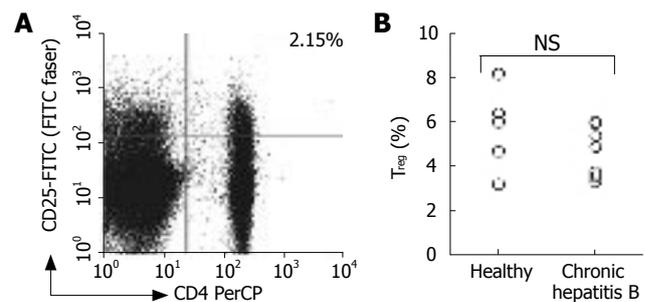


Figure 4 Comparison of CD4⁺CD25^{high} T cell population between patients with hepatitis B and healthy subjects. The cells that express CD4, CD25^{high} and CTLA-4 were identified by flow cytometry. Representative dot plots of an *ex vivo* sample of a patient with CHB is shown (A), numbers shown in the dot plot indicates percentage of cells in the quadrant lesion. Percentage of CD4⁺CD25⁺ T cells was shown for patients with CHB and healthy subjects (B).

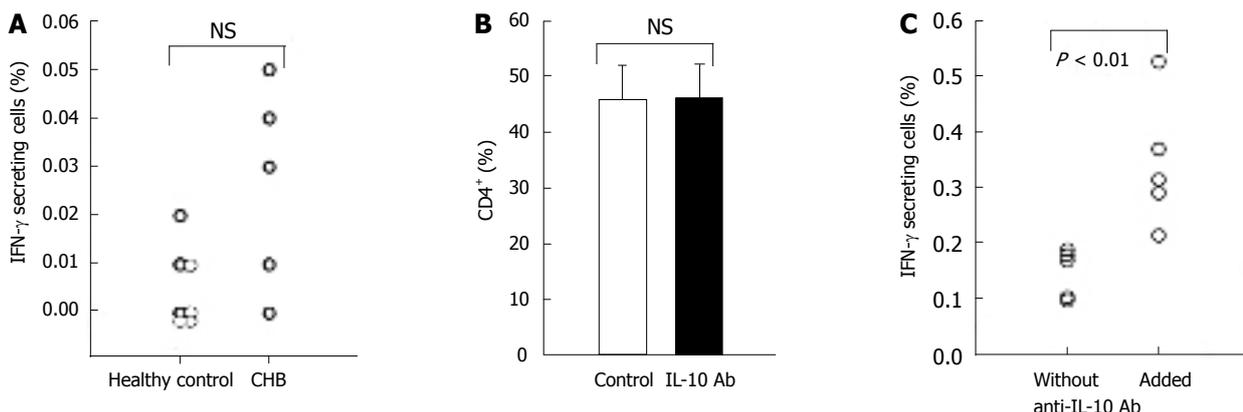


Figure 5 Addition of neutralizing anti-IL-10 antibody restores HBcAg-specific production of IFN- γ by CD4⁺ in patients with hepatitis B. PBMCs obtained from 5 patients with CHB and 7 healthy subjects were stimulated with HBcAg (10 μ g/mL) for 9 h and thereafter cells were stained for IFN- γ -secretion (PE) and anti-CD4-PerCP to determine the population of HBcAg-specific T_H1 being identified as IFN- γ ⁺ cells in CD4⁺ T cells (A). Anti-IL-10 neutralizing antibody or isotype-matched control antibody were added to the culture during stimulation with HBcAg. The addition of anti-IL-10 antibody did not affect the percentage of CD4⁺ T cells (B). In culture with anti-IL-10 antibody, numbers of HBcAg-specific T_H1 were significantly higher than those in culture with a control antibody (C).

antibody ($0.15\% \pm 0.04\%$, $P < 0.01$, Figure 5C).

T_{reg} depletion restores the response of IFN- γ -secreting CD4⁺ T cells to HBcAg

Similar to the effect of anti-IL-10 antibody, depletion of T_{reg} induced the recovery of HBcAg-specific T_H1 response. T_{reg} were depleted by a CD4⁺CD25⁺ T cell separation kit (Figure 6A) and the cultures were reconstituted by mixing separated fractions. T_{reg}⁻ culture contained 0.5% (mean) of CD4⁺CD25⁺ cells on average, while T_{reg}⁺ culture contained 3.5% of CD4⁺CD25⁺ cells on average (Figure 6B). The number of IFN- γ -secreting CD4⁺ cells in response to HBcAg significantly increased in T_{reg}⁻ culture by 6-fold ($0.03\% \pm 0.02\%$, mean \pm SD of 9 cases) compared with that in T_{reg}⁺ culture ($0.18\% \pm 0.05\%$, $P < 0.01$, Figure 6C).

Expression level of FOXP-3 and CTLA-4 was analyzed in 3 separate fractions to verify that CD4⁺CD25⁺ cells exhibited typical characteristics of T_{reg} cells. Fraction 3 (CD4⁺CD25⁺) expressed higher FOXP-3 than fraction 2 (CD4⁺CD25⁻) by 3.7 fold and fraction 1 (CD4⁻) by 7.8 fold. The percentage of total CTLA-4 expression in fraction 1, fraction 2 and fraction 3 was 0.45%, 2.71% and 32.71% respectively.

DISCUSSION

The response of T cells to HBcAg has been reported to contribute to the resolution and seroconversion of HBV infection in chronic hepatitis B^[31]. However, in the previous study we were unable to detect the recovery

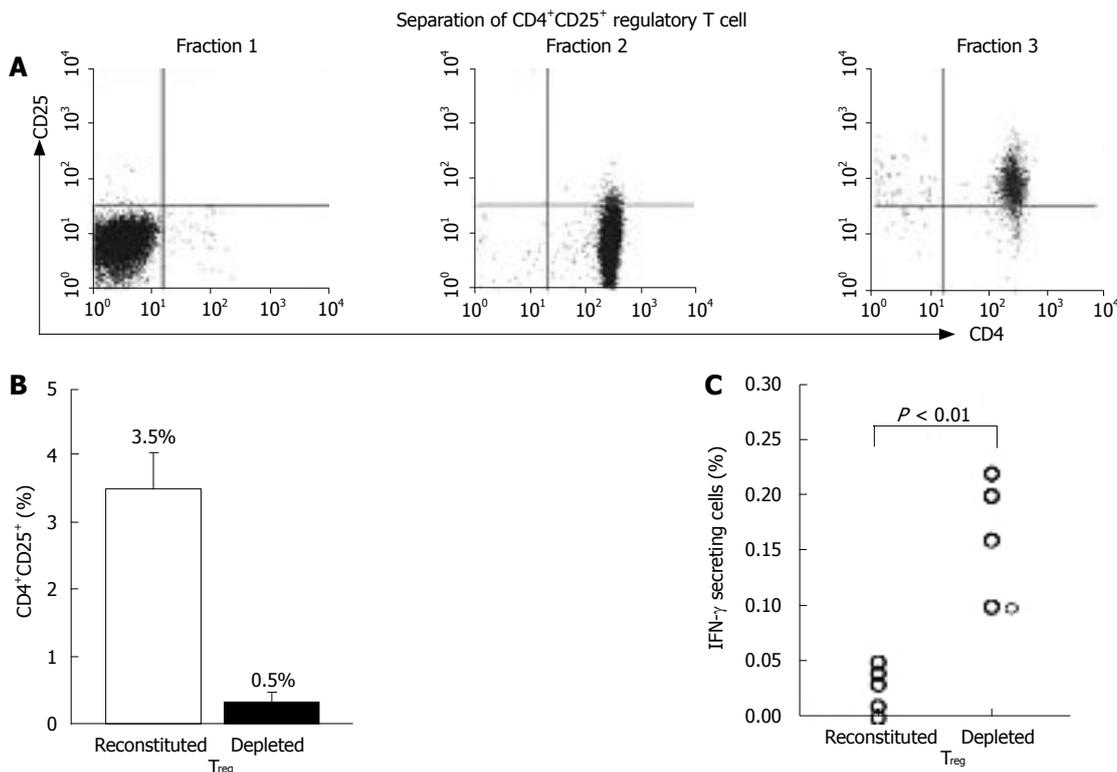


Figure 6 Depletion of CD4⁺CD25⁺ T cells from PBMCs increases HBcAg-specific production of IFN- γ in patients with hepatitis B. Using the differential expression of CD4 and CD25, cells were separated into 3 fractions; fraction 1 consisted of CD4⁺ cells, fraction 2 consisted of CD4⁺CD25⁺ cells and fraction 3 consisted of CD4⁻CD25⁺ cells (A). Thereafter, 2 sets of lymphocyte preparations were reconstituted by remixing fractions 1, 2 and 3 or by remixing fractions 1 and 2 (B). They were stimulated with HBcAg to finally stain a CD4⁺IFN- γ ⁺ population (C).

of HBcAg-specific T_{H1} despite the substantial increase in HBV-specific CTLs in patients receiving lamivudine therapy^[11]. The results raised a question about the profound suppression of CD4⁺ T cell response to HBV in patients with chronic hepatitis B. The current results showed that polarization of CD4⁺ T cells was suppressed when the cells were stimulated with HBcAg in patients with chronic hepatitis B. The mechanisms underlying this suppression of CD4⁺ T cells were through suppression of either direction to T_{H1} or T_{H2} by HBcAg stimulation, while HBsAg stimulation favored T_{H2} deviation in chronic hepatitis C.

It may be possible that T_{reg} cells are one of the mediators of the suppression of T_{H1} response to HBcAg as suggested by the results of an increased population of IL-10-secreting CD4⁺CD25^{high} cells. This indicates the presence of an inducible T_{reg} population which is specific for HBcAg and produces IL-10, as well as a natural T_{reg} population in patients with CHB. However, the role of HBcAg is controversial, since it can induce IL-18, a monokine that stimulates T lymphocytes and macrophages to produce IFN- γ , in both healthy subjects and patients with chronic hepatitis B^[32], and cause an increase in IL-10-producing T lymphocytes and monocytes *in vitro*^[33]. Our data indicate lack of HBcAg-specific T_{H1} response in CHB patients, although the results of IL-18 are not available. Our study was conducted on a small scale with 9 patients and the hyporesponsiveness of HBV-specific T cells should be investigated in studies with larger populations.

T_{reg} cells may be a common feature of immune sup-

pression in chronic viral infection. In HIV infection, appearance of T_{reg} in peripheral blood has been shown to have a suppressive role in CTL development against HIV antigen^[34]. In patients with chronic hepatitis C, the evolution of inducible T_{reg} cells specific for HCV antigens has been reported^[35] and the presence of CD8⁺ T_{reg} cells homing to suppress local inflammation in the liver has also been reported in HCV infection^[36]. Thus T_{reg} cells may have diverse effects during chronic viral infection; suppression of cellular immune response to eliminate the virus and the suppression of unfavorable tissue damage by the cellular immune response to the virus^[37]. In addition, there has been a report of different clinical features in patients with chronic hepatitis C, namely a higher prevalence of cryoglobulinemia in patients with lower T_{reg} cells^[38]. Although natural T_{reg} population may also contribute to the suppression of CD4⁺ T cell response from the results of CD4⁺CD25⁺-depletion, the population of CD4⁺CD25^{high} T cells *ex vivo* was not different between normal subjects (5.73% \pm 1.87%) and patients with chronic hepatitis B (4.73% \pm 1.15%) similar to the results of Franzese *et al.*^[39], while Stoop *et al.* have reported the increased T_{reg} population in peripheral blood of patients with CHB^[40]. The change in T_{reg} population and its contribution to pathogenesis needs to be evaluated by comparing various HBV diseases.

Manipulation of activity of T_{reg} cells specific for HBcAg may become one of the potent options in future therapy. An immunomodulating approach, which is indicated by successful use of GITR (glucocorticoid-

induced TNF-alpha receptors) to suppress activity of T_{reg} cells^[41], may become beneficial in patients with CHB.

In summary, this report demonstrates underlying mechanisms of suppression of immune responses to HBcAg in chronic HBV infection. A therapeutic approach to the molecules or cell types involved in these mechanisms may contribute to the improvement of prognosis in patients with chronic hepatitis caused by persistent replication of HBV.

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BASIC RESEARCH

Reversing gastric mucosal alterations during ethanol-induced chronic gastritis in rats by oral administration of *Opuntia ficus-indica* mucilage

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mucilage monosaccharides and membrane phospholipids, mainly PC and phosphatidylethanolamine (PE), may be the relevant features responsible for changing activities of membrane-attached proteins during the healing process after chronic gastric mucosal damage.

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Key words: Gastritis; Mucilage; Chronic gastric mucosal injury; Ethanol

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Abstract

AIM: To study the effect of mucilage obtained from cladodes of *Opuntia ficus-indica* (Cactaceae) on the healing of ethanol-induced gastritis in rats.

METHODS: Chronic gastric mucosa injury was treated with mucilage (5 mg/kg per day) after it was induced by ethanol. Lipid composition, activity of 5'-nucleotidase (a membrane-associated ectoenzyme) and cytosolic activities of lactate and alcohol dehydrogenases in the plasma membrane of gastric mucosa were determined. Histological studies of gastric samples from the experimental groups were included.

RESULTS: Ethanol elicited the histological profile of gastritis characterized by loss of the surface epithelium and infiltration of polymorphonuclear leukocytes. Phosphatidylcholine (PC) decreased and cholesterol content increased in plasma membranes of the gastric mucosa. In addition, cytosolic activity increased while the activity of alcohol dehydrogenases decreased. The administration of mucilage promptly corrected these enzymatic changes. In fact, mucilage readily accelerated restoration of the ethanol-induced histological alterations and the disturbances in plasma membranes of gastric mucosa, showing a univocal anti-inflammatory effect. The activity of 5'-nucleotidase correlated with the changes in lipid composition and the fluidity of gastric mucosal plasma membranes.

CONCLUSION: The beneficial action of mucilage seems correlated with stabilization of plasma membranes of damaged gastric mucosa. Molecular interactions between

INTRODUCTION

The *Opuntia ficus-indica* (*O. ficus-indica*) is a plant belonging to the Cactaceae family, located preferentially in arid zones^[1]. The main substance produced by this plant is mucilage composed mostly of water and polysaccharides, which may participate in adaptation mechanisms preventing dehydration or freezing^[2]. The mucilage obtained from cladodes of *O. ficus-indica* is soluble in water and produces colloidal solutions of high viscosity^[3].

The polysaccharide in mucilage with a high molecular weight^[4,5], is produced by specialized cells in the Cactaceae^[6]. Chemically, mucilage is composed of α -D-galactopyranosiluronic acid and β -L-rhamnopyranose, forming the main chain and β -D-galactopyranose, β -D-xylopyranose and α -L-arabinofuranose in the side chains^[7,8].

In Mexico, mucilage obtained from *O. ficus-indica* can cure topical inflammation and skin ulcerations^[9]. In addition, recent data suggest that mucilage derived from this plant can treat acute gastric damage. The anti-ulcer activity of mucilage from *O. ficus-indica* has been reported^[10]. The beneficial effect of cladodes from *O. ficus-indica* when simultaneously administered with ethanol seems related to an enhancement of gastric mucus production^[11]. Likewise, polygalacturonic acid^[4,7] and arabinogalactan protein in mucilage, probably act in combination with some protective factors including

macromolecules or small ligands of the gastric mucosa^[12]. Although these possibilities remain unproved, mucilage from *O. ficus-indica* could exert a cytoprotection as an anti-inflammatory agent, which can prevent rat gastric mucosal damage when administered concomitantly with the noxious agent^[13].

To solve the problem, we have developed a model of ethanol administration to rats, which could elicit a histological profile of gastric injury characterized by loss of surface epithelium and infiltration of polymorphonuclear leukocytes. This model produces evident alterations in plasma membranes of the gastric mucosa^[14]. After ethanol withdrawal^[15], these alterations may lead to increased membrane lipid peroxidation accompanying spontaneous restitution of the gastric mucosa epithelium.

Taking advantage of these histological alterations, as well as those in membrane lipid composition present in our model of chronic ethanol-induced rat gastric mucosal damage^[14], the present study assessed whether administration of mucilage extracted from *O. ficus indica* had a therapeutic effect on this experimental model of gastritis in rats.

MATERIALS AND METHODS

Preparation of mucilage

The cladodes were isolated from a cultivation of *O. ficus-indica* located in Milpa Alta village (near to Mexico City) and the identity of the plant was confirmed by the bibliographic data^[1]. Two kg of fresh cladodes free of spines was cut into cubes of 2 cm × 2 cm × 1 cm and placed in one liter of distilled water for 24 h. The supernatant was frozen and lyophilized, from which 41g of mucilage as a yellow-powder was obtained. A 0.25% solution of mucilage in water was prepared.

Animal treatment

A model of gastritis induced by chronic ethanol administration to rats has been described in detail elsewhere^[14]. Briefly, two groups of 20 and 15 male Wistar rats weighing 240-260 g, after an overnight fasting, received 1 mL of 50% ethanol through a gastric tube for one day and water containing 5% of ethanol *ad libitum* to achieve a daily intake of 9-10 g of ethanol per kg of body weight for five days. Then the animals developed gastritis. This was considered as time zero (once histological evidence of gastritis was achieved), at this time the ethanol ingestion was discontinued. The group of 20 animals was considered as the gastritis control group, which was divided into four subgroups of 5 animals. The first subgroup was studied at time zero whereas the second, third and fourth subgroups were studied after 24, 48, and 72 h, respectively. The group of 15 rats served as the gastritis group treated with mucilage (5 mg/kg of body per day, starting at time zero) and divided into three subgroups, 5 animals in each subgroup, and studied after 24, 48, and 72 h, respectively. The complementary control group of 15 healthy animals (divided into three subgroups, 5 animals in each subgroup), received only saline solution and was studied after 24, 48, and 72 h, respectively. The animals were killed by decapitation after administration of an overdose of

sodium pentobarbital. All the procedures were performed according to the Federal Regulations for *Care and Use of Experimental Animals* (Ministry of Agriculture and Animal Breeding; SAGARPA).

Histological assessment and subcellular fractionation of gastric mucosa

The stomach was removed from each rat, dissected along the greater curvature and rinsed in cold saline solution. Strips of gastric wall were embedded in paraffin, stained and analyzed as reported elsewhere^[14]. The remnant gastric tissue from the forestomach to the pylorus through the entire glandular mucosa, was homogenized in a buffer containing 0.25 mol/L of sucrose and 10 mmol/L of TRIS-HCl (pH 7.5). Plasma membrane was obtained from the whole homogenate by the method of Loten and Redshaw-Loten^[16], while the cytosolic fraction was obtained by differential centrifugation^[14]. Identity and purity of the subcellular fractions were routinely assessed by determining the activities of marker enzymes, namely 5'-nucleotidase and lactate dehydrogenase (LDH)^[14]. 5' nucleotidase was also used to assess the *in vivo* plasma membrane fluidity^[14].

Analytical procedures

In extracts of total lipids from isolated plasma membranes, phospholipid content was determined as previously described^[17]. Total cholesterol was determined with the colorimetric method described by Abell *et al.*^[18]. The cytosolic fractions were used as an enzyme source to determine the specific activities of LDH (EC 1.1.1.27) and alcohol dehydrogenase (ADH; EC 1.1.1.1) using the spectrophotometric methods of Vassault^[19] and Caballería *et al.*^[20] respectively. In all the assays, protein content was determined according to Lowry *et al.*^[21].

Statistical analysis

Statistical difference among groups was calculated by the two-way ANOVA test, and expressed as mean ± SE. In case of significance, student's-*t* test was applied.

RESULTS

Histological assessment of gastric samples from animals with gastritis and treated with mucilage

When compared to control rats (Figure 1A), at the onset of gastritis (T0), disrupted superficial epithelial cells, loss of specialized cells (glandular), slight submucosal edema, marked margination and infiltration of polymorphonuclear (PMN) leukocytes were found in gastric mucosa, proving clearly an inflammatory process (Figures 1B and 1C). The histological abnormalities generated by ethanol remained unchanged 24 h after spontaneous recovery (Figure 1C), but restoration of the gastric mucosa started 48 h after ethanol withdrawal. Therefore, three days after spontaneous recovery, a histological pattern corresponding to a moderate gastritis was observed in these animals (Figure 1D, Table 1). On the contrary, animals with gastritis and treated with mucilage showed an earlier recovery as reflected in a great amelioration of the inflammatory

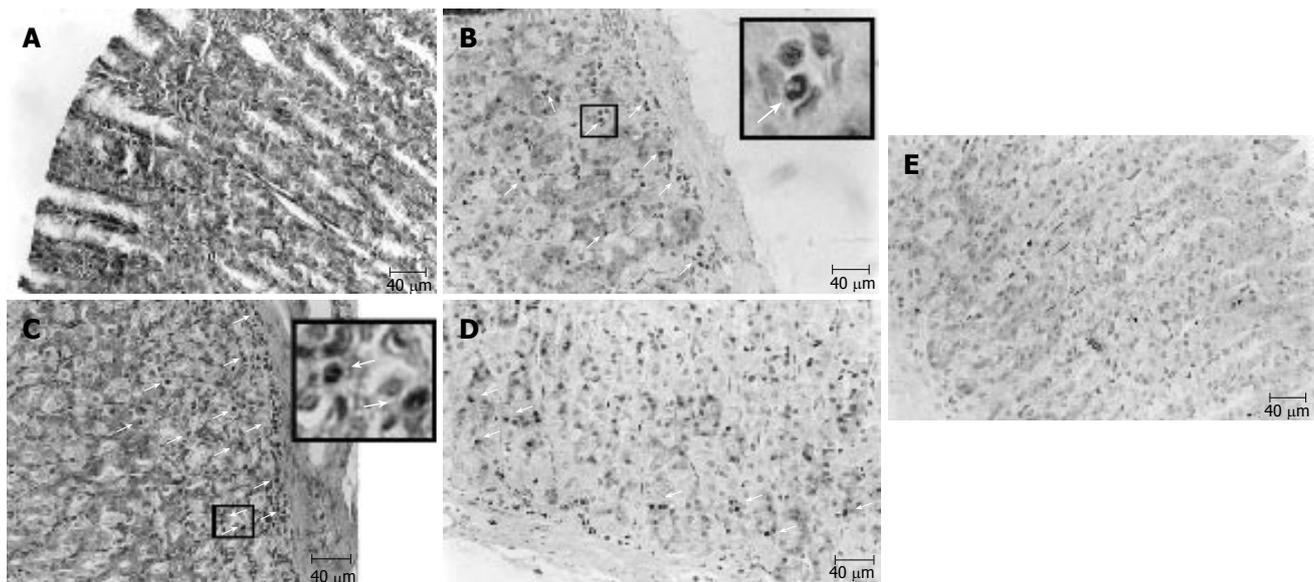


Figure 1 Representative micrographs of gastric mucosa of rats with gastritis and treated with mucilage. **A:** control animals; **B:** animals subjected to gastritis (T0); **C:** animals 24 h after ethanol withdrawal. In micrographs B and C, the presence of PMN infiltrate is shown by large arrows in the insets; **D:** histological profile of the spontaneous recovery of gastric mucosa 72 h after ethanol withdrawal (arrows: PMN); **E:** histological profile corresponding to similar conditions after treated with mucilage (3 doses).

Table 1 Incidence of microscopic lesions in stomach of rats treated with ethanol and mucilage from *O. ficus-indica*

Experimental groups	Time (h)	Parameters		
		S. epithelium (disruption)	Specialized cells (loss)	PMN infiltration
Controls	--	---	---	---
Gastritis + saline	0	+++	++/+++	+++
Gastritis + saline	24	+++	++	+++
Gastritis + saline	48	++/+++	+/++	+++
Gastritis + saline	72	+/++	+	+/++
Gastritis + mucilage	24	+++	++	+++
Gastritis + mucilage	48	++	+	+++
Gastritis + mucilage	72	+	--/+	+

Controls corresponding to rats treated with saline. Scale for injury degree: (-) absent, (+) slight, (++) moderate and severe (+++). PMN: Polymorphonuclear leukocytes; S.: Surface.

process (Figure 1E, Table 1).

Effects of mucilage on plasma membrane lipid composition and cytosolic activities of soluble enzymes in rats with chronic gastric mucosal injury

Along with the histological findings, the lipid composition of isolated mucosa plasma membranes in the experimental groups changed significantly. Table 2 depicts the phospholipid species found in plasma membranes obtained from mucosa of rats with gastritis and treated with mucilage. Chronic ethanol treatment (T0) had a dual effect on phospholipid distribution. Both phosphatidylinositol (PI) and phosphatidylcholine (PC) decreased significantly accompanying a progressive enhancement of phosphatidylethanolamine (PE) in plasma membrane. The less abundant phospholipid in plasma membranes of gastric mucosa was phosphatidic acid (PA). Likewise, the level of phosphatidylserine (PS) had no

Table 2 Phospholipid composition of stomach mucosal membranes of gastritis rats treated with mucilage from *O. ficus-indica* (mean \pm SE)

Experimental groups	Phospholipids (nmols of phosphate per mg of protein)				
	PA	PS	PI	PC	PE
Controls	29 \pm 4	103 \pm 12	172 \pm 9	464 \pm 25	206 \pm 20
Plus saline					
Gastritis (Time zero)	35 \pm 5	123 \pm 14	111 \pm 16 ^a	333 \pm 52	219 \pm 22
Gastritis (24 h)	34 \pm 5	121 \pm 12	139 \pm 10 ^a	314 \pm 37 ^a	321 \pm 32 ^a
Gastritis (48 h)	38 \pm 5	135 \pm 11	126 \pm 11 ^a	332 \pm 20 ^a	266 \pm 23
Gastritis (72 h)	40 \pm 5	141 \pm 19	126 \pm 15 ^a	372 \pm 30 ^a	247 \pm 18
Plus mucilage					
Gastritis (24 h)	38 \pm 4	136 \pm 11	148 \pm 21	321 \pm 35 ^a	347 \pm 33 ^a
Gastritis (48 h)	36 \pm 5	128 \pm 9	137 \pm 12 ^a	384 \pm 39	232 \pm 16
Gastritis (72 h)	29 \pm 4	102 \pm 19	160 \pm 15 ^b	453 \pm 31 ^b	196 \pm 18

PA: Phosphatidic acid; PS: Phosphatidylserine; PI: Phosphatidylinositol; PC: Phosphatidylcholine; PE: Phosphatidylethanolamine. ^a $P < 0.01$ vs controls; ^b $P < 0.01$ vs rats subjected to gastritis (untreated with mucilage).

significant change due to the presence of gastritis (Table 2). The ethanol treatment-induced alteration of PI and PC in plasma membrane persisted for up to 72 h after ethanol withdrawal. However, PE levels were normalized 48 h after spontaneous recovery. A single dose of mucilage (24 h) did not have any significant effect on ethanol-induced alterations in the lipid composition of gastric mucosal membranes, but subsequent administrations promptly normalized the content of phospholipids and their relative distribution in gastric membranes of animals undergoing gastritis (Table 2). Then, mucilage administration elicited a restoration of PC levels, close to the control levels (Table 2). Although mucilage exerted its effects on distribution of plasma membrane phospholipids during the treatment, neither the degree of mucosal damage nor the treatment with mucilage significantly modified the amount of total

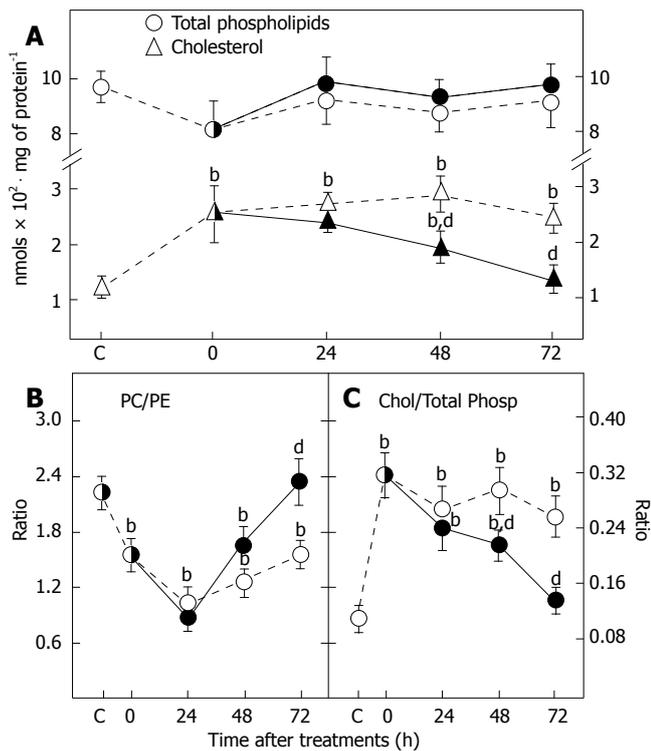


Figure 2 Total phospholipids and cholesterol in plasma membranes of mucilage-treated rats with gastritis (A) and the phosphatidylcholine (PC)/phosphatidylethanolamine (PE) methylation ratio (B) and cholesterol (chol)/total phospholipids (total phosp) ratio (C). In all cases mean \pm SE, $n = 5$. Animals subjected to gastritis are represented by empty symbols while those treated with mucilage are represented by solid symbols. ^b $P < 0.01$ vs healthy animal control. ^d $P < 0.01$ vs animals with gastritis not treated with mucilage.

membrane phospholipids (Figure 2A). However, plasma membrane cholesterol increased as a response to mucosal injury (Figure 2A). Thus, the group of rats subjected to gastritis showed an early increase of cholesterol content, which remained significantly higher than that in the control group during the experiment. In this context, administration of mucilage successfully restored the normal amount of cholesterol in mucosa plasma membranes (Figure 2A), and consequently, the cholesterol/phospholipids ratio was also modified (Figure 2C). This ratio was greatly enhanced in mucosal plasma membranes obtained from rats undergoing gastritis, and remained higher at all the tested time points. On the contrary, treatment with three doses of mucilage (72 h after ethanol withdrawal) normalized the cholesterol/phospholipids ratio (Figure 2C). The methylation index represented by the PC/PE ratio also decreased in animals with gastritis. This effect induced by ethanol, persisted even after 72 h of ethanol administration (Figure 2B). Interestingly, mucilage also corrected this parameter of the lipid composition of mucosal plasma membranes. This was more evident at the end of its administration to animals undergoing gastritis (Figure 2B). Therefore, alterations in the lipid composition of plasma membranes of injured gastric mucosae, were promptly normalized by the administration of mucilage.

Due to the effect of mucosal damage induced by ethanol, some cytosolic enzyme activities were significantly affected (Figure 3). As previously reported^[14], animals with gastritis showed a significant drop in ADH activity (54%).

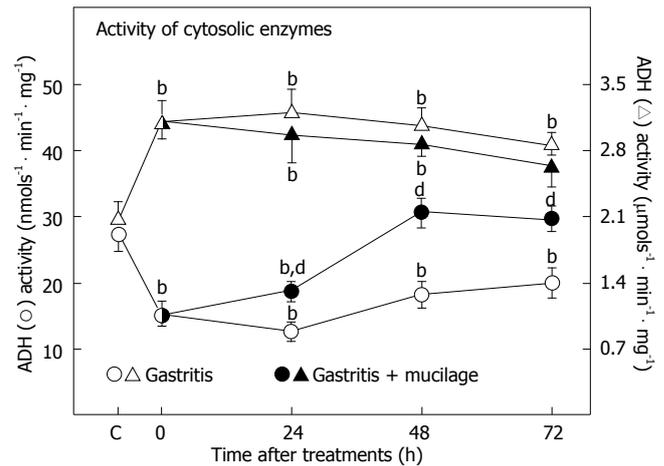


Figure 3 Effects of gastritis and mucilage from *O. ficus-indica* on cytoplasmic specific ADH and LDH activities. The results are expressed as mean \pm SE of five individual observations per experimental group for the alcohol (ADH) and lactate (LDH) dehydrogenases in the cytosolic fraction. Statistical significance is shown in Figure 2.

Regardless of the ethanol withdrawal, the activity of ADH remained abnormal during the tested recovery period (up to 72 h). However, mucilage promoted an early recovery of the ADH activity when administered to rats undergoing gastritis (Figure 3). The opposite to the LDH activity was observed. This cytosolic enzyme with a very high specific activity increased after chronic ethanol administration (133% over controls) and its activity remained significantly higher even 72 h after ethanol withdrawal. In this case, administration of mucilage also normalized LDH activity, but only after 3 doses (Figure 3), suggesting that administration of mucilage from *O. ficus-indica* to animals with ethanol-induced gastritis, could not only correct the alterations found in plasma membranes, but also practically normalize the activities of some soluble enzymes present in the cytosolic fraction obtained from the gastric mucosa.

Effects of gastritis and mucilage on membrane 5'-nucleotidase activity

The effects of gastritis and mucilage on the activity of 5'-nucleotidase were also evident. This ectoenzyme could respond to changes in the lipidic microenvironment, thus its activity could indirectly indicate the fluidity status of gastric mucosa plasma membranes. This enzyme presented a decreased activity at the onset of gastritis (T0) and was still lower 24 h after ethanol withdrawal (Figure 4, left panel). Thereafter, the activity of 5'-nucleotidase was suddenly enhanced and normalized after 72 h of spontaneous recovery. When rats undergoing gastritis were administered with mucilage, an early recovery of the plasma membrane activity of 5'-nucleotidase (24 h) was noted, which was followed by a sustained increase in the 5'-nucleotidase activity during the whole treatment period (Figure 4, left panel). The influence of the lipidic composition of plasma membranes on 5'-nucleotidase activity was evident when this enzymatic activity was plotted against indicative parameters of plasma membrane fluidity (Figures 2B and 2C). As shown in Figure 4B (right panel), the activity of 5'-nucleotidase correlated directly

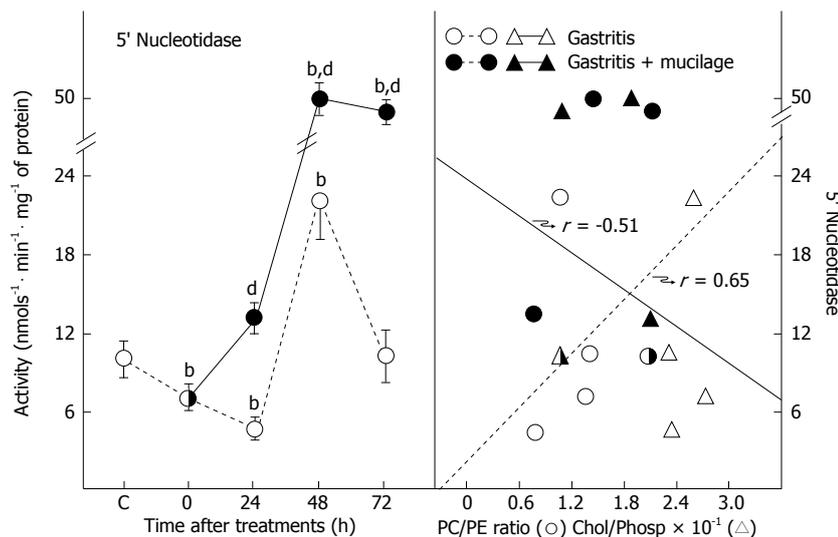


Figure 4 Activity of 5'-nucleotidase and its relation with the cholesterol/ phospholipid ratio in plasma membranes of mucilage-treated rats with gastritis. Left panel represents the mean \pm SE of five individual observations per experimental group for 5'-nucleotidase activity. The correlation between activity of this enzyme and the PC/PE ratio or the cholesterol (chol)/ phospholipid (phos) ratio is shown in the right panel of Figure 2. Statistical significance is indicated in the left panel of Figure 2.

with the PC/PE ratio ($r = 0.65$, $P < 0.005$), while increased cholesterol/phospholipids ratio negatively affected the activity of plasma membrane 5'-nucleotidase ($r = -0.51$, $P < 0.01$; Figure 4B, right panel).

DISCUSSION

Administration of concentrated ethanol solutions could induce damage to the gastric mucosa, including a disruption of up to 80% of the surface epithelial cells throughout the glandular stomach of rats^[22,23]. Indeed, ethanol administration could result in a reduction in resistance, transmucosal potential difference, H⁺ secretion, and increased appearance of Na⁺ in the lumen at neutral pH^[24,25], indicating that ethanol can induce profound changes in membrane permeability.

The present model resembles an active gastritis, similar to that found in humans. In fact, this model has been already validated by histological and biochemical findings^[14,15]. Furthermore, the main histological findings, such as inflammation and reduction of surface epithelial cells, as well as alterations in mucosal glands (Figure 1) and parietal cells^[26] are described in another model of chronic gastritis in rats^[27]. Our experimental model of ethanol-induced gastritis in rats is characterized by decreased PI and PC, and increased PE in plasma membranes of the gastric mucosa. PC is abundant in the gastric mucosa of rats, and the PC/PE ratio represents the lipid methylation activity^[28]. Additionally, the cholesterol/phospholipid ratio significantly decreased, strongly suggesting that ethanol-induced gastritis diminishes the membrane permeability^[14]. In fact, the cholesterol/phospholipid ratio influences the fluidity of a variety of membranes, and increases during the occurrence of altered membrane permeability^[29,30]. All these modifications in the membrane lipid composition of gastric mucosa obtained from animals with gastritis are due to the changes in the rate of lipid peroxidation^[15].

Lipid peroxidation and its effects on the composition and function of plasma membranes seem to play their part in chronic ethanol-induced gastric mucosa injury, promoting diminished binding of ligands to membrane histaminergic H₂-receptors, which is inversely correlated

with the rate of restoration of the surface epithelium in gastric mucosa^[13]. In this regard, the effects of administration of mucilage obtained from *O. ficus-indica* on gastric mucosal lesions need to be further studied.

In the present model, mucilage administration was capable of correcting the alterations found in plasma membrane, especially its lipid composition, suggesting that mucilage can accelerate the repair of gastric mucosal lesions in rats undergoing gastritis (Figures 1-3). Histological examination revealed that the main action of mucilage in injured gastric mucosa was an anti-inflammatory effect, which seemed to be involved in the further restitution of the mucosal integrity. Along with this, mucilage contains polygalacturonic acid^[4] and arabinogalactan, which could interact with macromolecules or small ligands of the gastric mucosa, enhancing gastric mucus production^[11].

In addition to the partial blocking effect on the PMN infiltrate, the specific activity of cytosolic enzymes ADH and LDH also normalised after the treatment with mucilage. These enzymes seem related to the condition of the gastric mucosa in some extent, since altered LDH and ADH activities seem to be associated with processes of gastric carcinogenesis and intestinal metaplasia^[31,32].

The change of phase from gel to liquid of membrane phospholipids can result from an abnormal increase of permeability and loss of cellular functionality. In this sense, polysaccharides may stabilize and protect the biological membranes, allowing the transitional phase remaining within the range of biological activity, and avoiding the damage induced by dehydration or freezing^[2]. Polysaccharide interaction between κ -carrageenan^[33] or thehalose^[34] or fructanes^[35] and the polar heads of phospholipids, plays a role in stabilizing membranes. Saccharides interact with proteins such as mucin, and/or the polar heads of membrane phospholipids. Therefore, they could originate a protective effect once they replace hydrogen bonds of water molecules, generating and increasing local viscosity^[36]. This can avoid dehydration such as that produced by alcohol. The changes observed in cell membrane after chronic ethanol treatment, such those in the lipid composition, could decrease or even induce a

complete loss of the selective membrane barrier function which could eventually lead to cell death.

Alterations of gastric mucosal cell membranes seem correlated with the activity of 5'-nucleotidase, which permits monitoring the *in vivo* changes in the membrane lipid composition after mucilage treatment. The effects of gastritis and mucilage on the 5'-nucleotidase activity deserve some comments. Changes in the lipid composition and the cholesterol/phospholipid molar ratio differentially affect the activities of 5'-nucleotidase, Mg²⁺-ATPase, and γ -glutamyltransferase^[37]. Since 5'-nucleotidase is attached to the membrane bilayer through a glycosylphosphatidylinositol anchor, PC and PE can induce the highest degree of activation of 5'-nucleotidase^[38]. Ethanol alone (up to 400 mM) does not affect the 5'-nucleotidase activity, but its derivative phosphatidylethanol, enhances 5'-nucleotidase activity^[39]. The present data indicate that changes found in the activity of this enzyme reflect changes in the lipidic microenvironment of gastric plasma membranes.

On the other hand, the activity of ecto-5'-nucleotidase constitutes a key enzyme responsible for adenosine production in rat hearts^[40]. In the rat stomach, adenosine has been demonstrated to inhibit gastric acid secretion probably by indirectly inhibiting gastrin release, which seems to be regulated through participation of A(1)-adenosine receptors^[41]. Adenosine also increases somatostatin release by acting on A(2A) receptors^[42] and may have a putative gastroprotective effect against several types of inductors of gastric mucosal injury such as deficient circulatory conditions^[43] and indomethacin-induced gastric lesions^[44], as well as against stress- and ethanol-evoked gastric lesion formation^[45]. Whether endogenous increase of gastric mucosal levels of adenosine, mediated by mucilage-induced activation of 5'-nucleotidase, participates in the beneficial action of mucilage, remains to be clarified.

In any case, the association between mucilage polysaccharides and membranes may contribute to the repair of damaged membranes^[2]. This may occur following the insertion of polysaccharides into membranes, originating a lateral spacing among the phospholipid polar heads, reducing the van der Waals interactions within the hydrocarbon chains, increasing viscosity and reducing mobility around the disturbed membrane. All these changes accompany capturing and associating water molecules^[2].

In conclusion, mucilage may exert its anti-inflammatory effect by promoting the healing process of gastritis in at least three ways: formation of a viscous protective cover against the damage induced by ethanol or other noxious substances; restoration of stomach epithelial surface and stabilization of plasma membranes; participation in enzyme recovery, restoration of both the cholesterol/phospholipid ratio and membrane fluidity.

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Assessment of liver fibrosis by a noninvasive method of transient elastography and biochemical markers

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the existing LF markers.

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Abstract

AIM: To assess the correlation between the fibrotic area (FA) as calculated by a digital image analysis (DIA), and to compare the diagnostic accuracy of FibroScan to the other existing Liver fibrosis (LF) markers using the receiver operating curve analysis.

METHODS: We recruited 30 patients who underwent a liver resection for three different etiologies including normal liver, hepatitis B, and hepatitis C. Liver stiffness was measured by using a FibroScan. The FA was then calculated by DIA to evaluate LF in order to avoid any sampling bias.

RESULTS: The FA negatively correlated with Prothrombin time (PT), platelet count, lecithin-cholesterol acyltransferase (LCAT), and pre-albumin (ALB). On the other hand, the findings of FibroScan correlated with similar markers. The FA positively correlated with FibroScan, serum hyaluronate level, and type IV collagen level, and aspartate transaminase to platelet ratio index (APRI). The area under the receiver operating curve for FibroScan was higher than that for the other markers, even though the statistical significance was minimal.

CONCLUSION: Our findings suggest that FibroScan can initially be used to assess LF as an alternative to a liver biopsy (LB) and serum diagnosis, because it is a safe method with comparable diagnostic accuracy regarding

INTRODUCTION

Liver fibrosis (LF) is characterized by the accumulation of an extracellular matrix, which distorts the hepatic architecture^[1,2]. The major etiologies of LF are viral-associated hepatitis, alcohol abuse, non-alcoholic steatohepatitis and autoimmune disease. The progression of LF increases the stiffness of liver and the resistance of liver blood flow^[1,3]. An insufficiency of liver blood flow results in liver failure and eventual liver cirrhosis. Once cirrhosis develops, liver transplantation is the only therapy to avoid a fatal condition^[4]. Therefore, an accurate assessment LF is very important in order to predict the prognosis and start the appropriate prophylactic therapy to prevent disease progression.

Liver biopsy (LB) is still the gold-standard method for assessing LF^[1,2,5]. However, it is difficult to perform LB for all patients who need to be assessed repeatedly due to its invasiveness and prohibitive cost. In addition, biopsy samples are usually too small to diagnose the disease accurately and diagnostic opinions often differ among pathologists^[6,7]. As a result, a pathological examination does not always provide an accurate diagnosis. Furthermore, histological quantification of LF is also difficult because of the diagnostic variability^[2,8]. Some studies have shown that the diagnostic accuracy of a digital image analysis (DIA) for LF is more reliable than histological scoring systems^[8-10] such as METAVIR and Ishak score. Therefore, we assessed LF using DIA, which could accurately calculate the fibrotic area (FA), in order to avoid diagnostic variability

and errors.

The liver plays a very important role in maintaining such serum proteins as albumin, cholesterol and coagulation factors. The production of such proteins decreases in liver cirrhosis^[11-13]. Although these protein levels decrease in the late stage of liver cirrhosis, it is still not fully understood which markers represent liver damage in the early stage or which liver functional marker is correlated with LF.

Recently, transient elastography (FibroScan[®]; Echosens, Paris, France) has become available for the assessment of LF as a rapid noninvasive method, which can measure liver stiffness from outside of the body^[14-17]. FibroScan has been compared to such classical markers as hematological test [the aspartate transaminase to platelet ratio index (APRI)] and fibrotest, accurately representing the state of liver fibrosis evaluated by METAVIR scoring system. The diagnostic accuracy of FibroScan has thus been found to be comparable with that using traditional markers.

In the present study, we selected patients who underwent a hepatectomy for this study. In this setting, a large amount of tissue could be used for analysis in order to reduce the risk of sampling bias. In addition, three different backgrounds of liver disease could be evaluated in order to make an accurate diagnostic value of FibroScan for LF without any sampling bias. We first measured the FA precisely using DIA to avoid any diagnostic variability. We next evaluated the correlation between FA and multiple biochemical markers to identify which markers could represent a deterioration of liver function associated with FA. In addition, we also evaluated the correlation between FibroScan and multiple biochemical markers to see whether FibroScan could accurately represent liver function. Furthermore, we also evaluated the correlation between FA and existing LF markers including FibroScan, hyaluronate, type IV collagen, and APRI. The final aim of this study was to assess the correlation of our findings to FA and to compare the clinical diagnostic accuracy of FibroScan and other markers.

MATERIALS AND METHODS

Patients

We examined 30 patients who underwent a liver resection from January 2003 to May 2005. We recruited 10 patients each with three different etiologies including a normal liver, hepatitis B, and hepatitis C. The pathological diagnosis for the liver was made in all 10 samples of normal liver, liver fibrosis, and liver cirrhosis. The indications for a hepatectomy in the normal liver were metastatic liver tumor while the indications for those with viral hepatitis were hepatocellular carcinomas. Informed consent was obtained from each patient included in this study and the study design conformed to the ethical guidelines of the Declaration of Helsinki as reflected in a priori approval by the institution's human research committee.

Liver stiffness measurement

The principle of elastography using a FibroScan[®] (Echosens) has been described previously^[14]. All measurements were performed in the right lobe of the liver through the

intercostal spaces in patients lying in the dorsal decubitus position with their right arm in maximal abduction. The tip of the probe transducer was covered with coupling gel and placed on the skin between the ribs at level of the right liver lobe. The operator, assisted by ultrasound time-motion and A-mode images provided by the system, located a portion of the liver that was at least 6 cm thick and free of any large vascular structures. Once the area of measurement was located, then the operator pressed the probe button to begin image acquisition. The measurement depth ranged from 25 mm to 45 mm and 10 validated measurements were performed in each patient. The success rate was calculated as the number of validated measurements divided by the total number of measurements.

The results were expressed in kPa. The median value was considered representative of the elastic liver modulus. The whole examination lasted less than 5 min. Only procedures with 10-validated measurements and a success rate of at least 60% were considered reliable.

Digital image analysis of liver fibrotic area

Liver fibrosis was evaluated using a computer program (NIH Image V1.62, National Institutes of Health, Bethesda, MD). Tissue sections were examined with Azan-Mallory staining. The fibrotic area was stained blue and depicted as the only blue signal using Adobe Photoshop CS (Adobe Systems Incorporated, San Jose, CA). Each patient was examined with 15 different fields in 3 different specimens. One field contained at least 10 portal tracts. The area of blue signals was calculated.

Statistical analysis

A statistical analysis of the relationship between pathological fibrosis and other clinical data was performed with Spearman's rank correlation coefficient using the StatView 4.5 software package (Abacus Concepts Inc., Berkeley, CA). The area under the receiver operating characteristic curve (AUC) analysis was performed using the MedCalc software package (Ver 8.0.1.0, Mariakerke, Belgium). All results are expressed as mean \pm SD. $P < 0.05$ was considered statistically significant.

RESULTS

We selected patients who underwent a hepatectomy with three different etiologies (Table 1). A large enough specimen for histological assessment could only be obtained from a surgical specimen since biopsy specimens are often insufficient to make an accurate diagnosis. We assessed the exact fibrotic area (FA) of LF using a digital image analysis (DIA), which is a simple and reliable method. We depicted only the blue signals from the full color image, and calculated FA. Representative photographs of the Azan-Mallory stain and the blue signals after depiction are shown in Figure 1. This method could thus be used for evaluating the pathological FA without diagnostic bias.

The characteristics of the patients are shown in Table 1. The liver stiffness was measured by FibroScan in all patients. We also measured serum hyaluronate, type IV collagen, and HGF levels in addition to performing routine

Table 1 Characteristic of the patients

Characteristics		n = 30	
Etiology (NBNC:B:C)	10:10:10	Choline esterase (IU/L)	262.80 ± 69.34
Background (N:CH:LC)	10:10:10	Pre-ALB (mg/dL)	21.92 ± 7.01
Sex (M:F)	22:8	RBP (mg/dL)	3.19 ± 1.39
Age (yr)	65.4 ± 10.3	LCAT (U)	100.66 ± 25.78
BMI	24.3 ± 3.1	Apo-A1 (mg/dL)	136.73 ± 32.43
ALB (mg/dL)	4.03 ± 0.60	ICGR ₁₅ (%)	12.86 ± 6.44
Total Bilirubin (mg/dL)	0.81 ± 0.28	Fibrotic area (%)	15.32 ± 9.69
PT (%)	98.83 ± 16.85	APRI	0.69 ± 0.46
PT-INR	1.01 ± 0.16	FibroScan (kPa)	15.47 ± 13.02
AST (IU/L)	41.63 ± 21.56	Hyaluronate (ng/mL)	120.50 ± 87.71
ALT (IU/L)	37.03 ± 21.58	Type IV collagen	6.85 ± 5.11
Platelet count (10 ⁴ /μL)	16.14 ± 4.57	HGF (ng/dL)	0.30 ± 0.09
ZTT (K-U)	13.57 ± 9.96	TTT (M-U)	7.56 ± 6.08
GGT (IU/L)	82.47 ± 73.13		

BMI: Body mass index; ALB: Albumin; PT: Prothrombin time; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ZTT: Zinc turbidity test; TTT: Thymol turbidity test; GGT: Gamma glutamyl transpeptidase; APRI: AST-to platelet ratio index; HGF: Hepatocyte growth factor; RBP: Retinol-binding protein; LCAT: Lecithin-cholesterol acyltransferase; Apo: Apolipoprotein; ICG: Indocyanine.

Table 2 Correlation with fibrotic area

Markers	r	P values
ALB	-0.388	0.067
T-Bil	0.13	0.559
PT	-0.459	0.026 ^a
PT-INR	0.472	0.022 ^a
Platelet count	-0.58	0.003 ^a
RBP	-0.515	0.011 ^a
Pre-ALB	-0.609	0.002 ^a
CholE	-0.299	0.168
LCAT	-0.447	0.032 ^a
ApoA	-0.045	0.839
ICGR ₁₅ (%)	0.364	0.088
AST	0.295	0.173
ALT	0.243	0.266
ZTT	0.373	0.079
G-GT	0.068	0.762

r > 0.4 or r < -0.4 was considered to indicate a correlation with the fibrotic area. ^aP < 0.05 was considered to be significantly different.

chemical laboratory tests. Furthermore, we measured pre-albumin (ALB), retinol-binding protein (RBP), lecithin-cholesterol acyltransferase (LCAT), Apo AI, and indocyanine green (ICG) R₁₅ levels to investigate the relationship between LF and liver function. The patients enrolled for this study showed a mixed etiology and various types of liver damage. However, the mean value of all biochemical data showed a nearly normal range, indicating that the sample bias might be minimal. Among the examined biochemical data, PT-INR, platelet count, RBP, pre-ALB, and LCAT levels correlated with FA (Table 2). On the other hand, ALB, platelet count, pre-ALB, LCAT levels and ICGR₁₅ all correlated with the FibroScan findings (Table 3). A linear regression analysis revealed two types of correla-

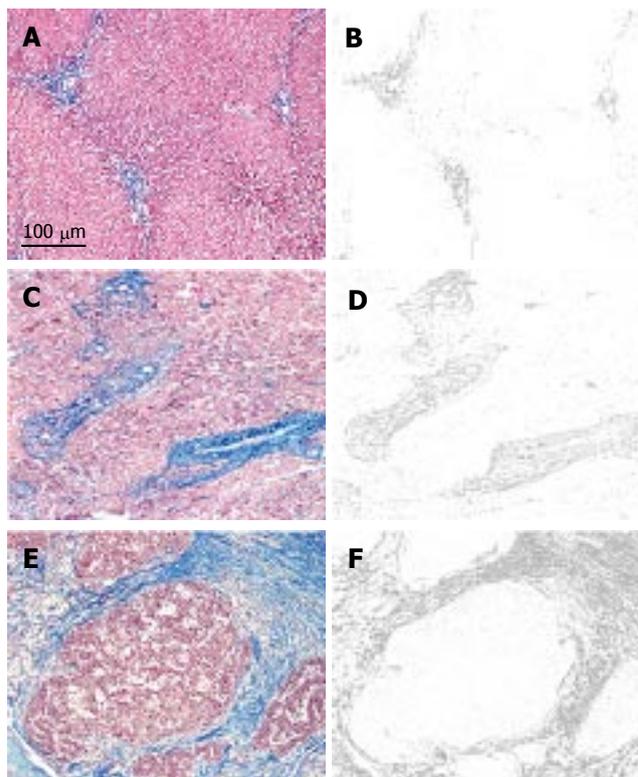


Figure 1 Hematoxylin-eosin staining of the surgical specimens (A, C, and E). The images scanned and depicted only blue signals using a computer program. The blue signals were calculated as the area of fibrosis (B, D, and F). The results of such fibrosis were 3.2% for B, 12.2% for D, and 27.7% for F. The bar shows 100 μm.

Table 3 Correlation with FibroScan

Markers	r	P values
ALB	-0.446	0.013 ^a
T-Bil	0.127	0.506
PT	-0.144	0.452
PT-INR	0.088	0.647
Platelet count	-0.431	0.017 ^a
RBP	-0.351	0.057
Pre-ALB	-0.535	0.002 ^a
CholE	-0.179	0.347
LCAT	-0.413	0.023 ^a
ApoA	-0.25	0.184
ICGR ₁₅ (%)	0.45	0.012 ^a
AST	0.21	0.268
ALT	-0.001	0.998
ZTT	0.577	0.006 ^a
G-GT	0.118	0.538

r > 0.4 or r < -0.4 was considered to indicate a correlation with the fibrotic area. ^aP < 0.05 was considered to be significantly different.

tions between FA and the findings of a biochemical analysis. One showed a negative correlation, which was seen for ALB, pre-ALB, LCAT, and platelet count (Figures 2 A-D). The other showed a positive correlation to the FibroScan findings, which was seen for ZTT and ICGR₁₅ (Figures 2 E and F). The pre-ALB levels showed the highest correlation with both FA and FibroScan among the examined biochemistry findings.

We compared the correlation between FA and existing LF markers including FibroScan, hyaluronate, type IV

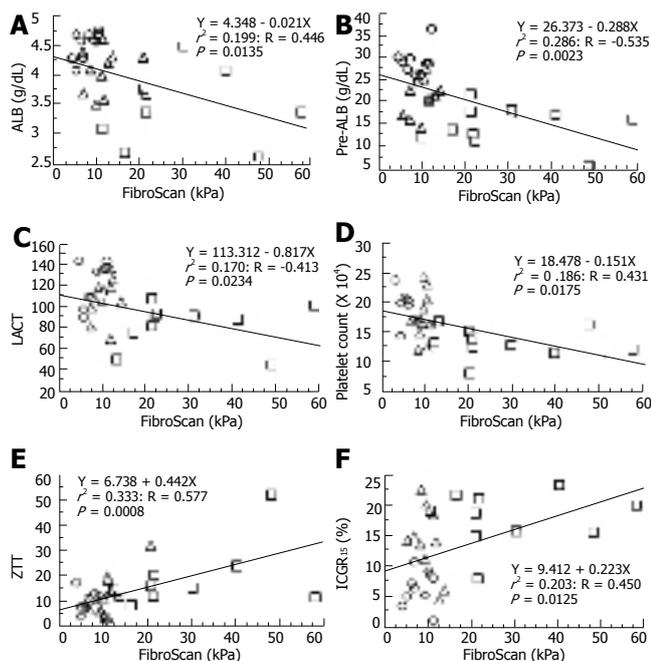


Figure 2 A linear regression analysis of FibroScan and clinical laboratory tests including liver functional markers. A negative correlation was seen for ALB (A), pre-ALB (B), lecithine-cholesterol acyltransferase (LCAT) (C), and platelet count (D). On the other hand, a positive correlation was seen for ZTT (E) and ICGR15 (F). ○: NBNC and normal liver; △: HBV and chronic hepatitis; □: HCV and liver cirrhosis.

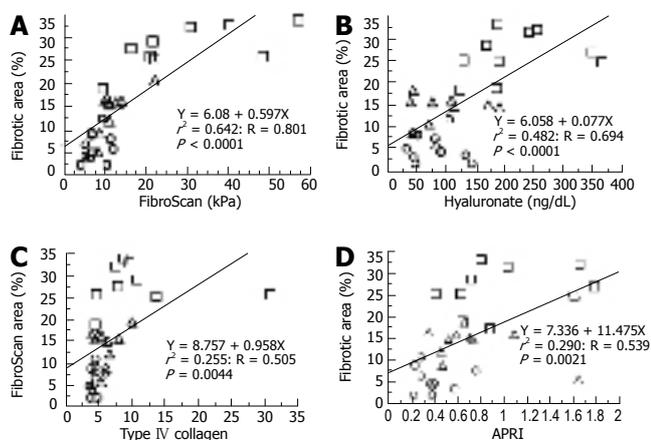


Figure 3 A linear regression analysis of the fibrotic area and liver fibrotic markers including FibroScan. A positive correlation was seen for all the markers such as FibroScan (A), hyaluronate (B), type IV collagen (C), and aspartate transaminase levels in comparison to the platelet ratio index (APRI) (D). ○: NBNC and normal liver; △: HBV and chronic hepatitis; □: HCV and liver cirrhosis.

collagen, and APRI (Figure 3). All markers correlated with FA. The correlation with the FibroScan findings was much higher than that with any other markers, even though the serum hyaluronate level was formerly believed to be the best available marker for evaluating LF^[8,18,19].

We investigated the area under the receiver operating curve (AUC) in order to compare the diagnostic accuracy of FibroScan and other markers (Table 4). The diagnostic specificity of FibroScan increased when the FA increased. The AUC of FibroScan was the highest among the markers at any level of FA, even though the statistical difference was minimal.

Table 4 Comparison of diagnostic accuracy of FibroScan and other markers

Fibrotic Area > 10%						
	OC	Sens	Speci	SE	95% CI	AUC
FibroScan	9.1	100	76.9	0.047	0.777 - 0.999	0.932
HA	96.0	76.5	84.6	0.080	0.618 - 0.924	0.803
Col	6.6	52.9	100	0.081	0.615 - 0.923	0.801
APRI	0.57	76.5	84.6	0.081	0.615 - 0.923	0.801
Fibrotic Area > 20%						
	OC	Sens	Speci	SE	95% CI	AUC
FibroScan	13.6	100	95.5	0.024	0.868 - 1.000	0.991
HA	106.0	100	77.3	0.061	0.797 - 0.993	0.946
Col	6.6	87.5	90.9	0.071	0.758 - 0.985	0.918
APRI	0.58	87.5	68.2	0.098	0.642 - 0.937	0.824

OC: Optimal cut off level; Sens: Sensitivity; Speci: Specificity; SE: Standard error; CI: Confidence interval; AUC: Area under the curve; HA: Hyaluronate; Col: Type IV collagen; APRI: AST-to platelet ratio index.

DISCUSSION

We found that the biochemical data correlated with FA based on DIA. Among the examined markers, pre-ALB showed a higher negative correlation to FA and FibroScan than the other markers. The diagnostic accuracy of FibroScan was higher than that of the other existing markers although the statistical significance was minimal. FibroScan can therefore be used to evaluate LF and liver function without any unnecessary invasiveness.

Biochemical assessment of LF

There are many ways to assess LF by serum biomarkers and pathological evaluations^[1,8,18-22]. Most such studies have been designed to evaluate fibrosis in chronic hepatitis C patients. Simple biomarkers such as those for aspartate aminotransferase (AST)/alanine aminotransferase (ALT) ratio (AAR) and AST-to platelet ratio index (APRI), and commercial tests such as Fibrotest[®] (Biopredictive, Paris, France) have been validated to distinguish between patients with severe LF and a normal liver. However, it is unclear whether they represent FA accurately regarding liver disease with various etiologies. APRI correlated with FA in our study. However, the relative factor was much less than observed for the other markers. It is easy to examine APRI in clinical examinations. However, it may not accurately represent FA until the liver disease has already reached a severely advanced stage. Therefore, the clinical benefit of the existing markers or their combinations may be limited by the etiology or stage of the disease. On the other hand, hyaluronate and type IV collagen were significantly correlated with FA in our results. Hyaluronate and type IV collagen are deposited in perisinusoidal lesions and can be used as specific markers to detect liver fibrosis^[8,18-20]. In addition, the serum levels both in liver cirrhosis and in fibrosis have been found to correlate with severity of the disease^[21,22]. In fact, they demonstrated a good correlation with FA in our results although AUC of them decreased in fewer FAs. Our findings suggest that hyaluronate and type IV collagen can thus be used to assess severe LF or cirrhosis. However, it would be difficult to assess LF at its early stage.

Liver stiffness as a novel marker for assessment of LF

Recently, FibroScan has been tested for assessment of LF in comparison to the classical markers^[14-17]. The diagnostic accuracy of FibroScan increases when it is used for severe fibrosis, whereas it decreases when it is used for a nearly normal liver. In our study, the diagnostic accuracy of FibroScan for livers with more than 20% FA was higher than that for livers with 10% FA. Our optimal cut-off level for FibroScan was 13.6 kPa, which could thus distinguish livers with more than 20% FA. This result is consistent with those in recent reports^[15,16] investigating viral hepatitis C patients with severe fibrosis or cirrhosis. Although AUC of the tested markers for more than 20% FA was higher than that for more than 10% FA, AUC of FibroScan for more than 10% FA maintained a fairly high level. Therefore, our results suggest that FibroScan is useful and reliable for assessment of earlier fibrotic stage compared to other existing markers and can be used for assessment of LA. However, these findings should be tested in prospective studies using a large number of cases because our results are based on a preliminary study. Especially, reduction of AUC for fewer FAs implies limitation of the diagnostic value of FibroScan in early LF stage.

Why AUC of FibroScan for fewer FAs decreases may be due to the system itself. Elasticity of the liver can be calculated by the different velocities between ultrasound (5MHz) and low-frequency (50 Hz) elastic waves^[17]. Wave velocity in liver was affected by fibrosis and watery distributions in liver^[23]. In general, watery distributions in the organ could be altered in the general systemic condition of the body such as inflammation which induces organ edema^[24]. In addition, watery contents in earlier LF stage may alter due to active hepatitis. On the other hand, watery distributions in late LF stage may be more stable than that in its earlier stage. Liver atrophies in severe LF and cirrhosis may reduce the space to store watery contents. One dimensional transient method could not eliminate the bias of watery distributions in liver, which may affect velocity and elasticity. However, we found that FibroScan could be used for primary assessment of LF among the existing non-invasive markers.

DIA for accurate assessment of LF

Quantification of LF is very important for detecting severity of the disease. There are several scoring systems, such as histology activity index of Knodell *et al.*, and its modification by Ishak *et al.*, and METAVIR system^[5]. However, such diagnostic variability cannot be ignored in pathological examinations due to the bias of pathologists and small samples^[7,8]. Therefore, digital image analysis should be performed for quantification of LF^[8]. On the other hand, the clinical benefits of digital image analysis remain controversial^[25]. The problem of this approach is the methodology used to depict fibrous signals. Once original pathological images are converted into gray scale, it becomes very difficult to distinguish between stained and non-stained areas for specific types of staining. In fact, a histogram of the depicted images can show overlapping curves between them^[25]. Therefore, specific fibrotic signals should be depicted in a full color image in order to eliminate any other colored signals, which could thus be

other cellular components. In this setting, the area of LF could be assessed precisely reflecting the actual state of FA. Whether this strategy can be used for small pathological specimens from LB remains unclear. We used large samples after surgical removal of the tissue to eliminate any sampling errors due to a small size. Therefore, the usefulness of DIA for LB specimens should be evaluated in other settings in future.

Biochemical markers for the liver function show a negative correlation to LF

Liver function deteriorates during progression of LF and cirrhosis. However, whether serum proteins decrease with severity of the disease has not been fully understood and whether they are markers for LF remain unclear. Child-Turcotte-Pugh (CTP) score is a classical liver functional indicator, which comprises serum albumin level, bilirubin, prothrombin time, and other clinical signs^[26]. However, CTP score in early stage of the disease does not show alteration^[26,27] along with the disease progression, suggesting that factors in CTP score are stable in early LF stage. On the other hand, we found that LCAT and pre-ALB significantly correlated with LF. Previous reports indicate that LCAT^[28,29] and pre-ALB^[30] correlate with severity of the disease and galactose elimination test, respectively. Our results showed that LCAT and pre-ALB were significantly correlated with FA assessed by DIA for LF, suggesting that LCAT and pre-ALB are markers for detecting LF. Although our study was a preliminary one using only a small number of patients, the results nevertheless support the findings of previous reports.

In conclusion, we assessed FA using DIA to eliminate the diagnostic variability often observed in pathological specimens. We examined the diagnostic accuracy of FibroScan, which was compared to that of simple biomarkers used in routine laboratory tests and extracellular matrix markers. FibroScan therefore showed a better correlation with FA than the existing LF markers, suggesting that FibroScan can be used as an alternative to LB in assessment of LF, as it is safe and has a sufficient diagnostic accuracy.

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Cortex cinnamomi extract prevents streptozotocin- and cytokine-induced β -cell damage by inhibiting NF- κ B

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Abstract

AIM: To clarify the mechanism underlying the anti-diabetic activities of *cortex cinnamomi* extract (CCE).

METHODS: To induce *in vivo* diabetes, mice were injected with streptozotocin (STZ) *via* a tail vein (100 mg STZ/kg body weight). To determine the effects of CCE, mice were administered CCE twice daily for 7 d by oral gavage starting 1 wk before the STZ injection. Blood glucose and plasma insulin concentration were measured as an index of diabetes. Also, to induce cytotoxicity of RINm5F cells, we treated with cytokines (IL-1 β (2.0 ng/mL) and IFN- γ (100 U/mL)). Cell viability and nitric oxide production were measured colorimetrically. Inducible nitric oxide synthase (iNOS) mRNA and protein expression were determined by RT-PCR and Western blotting, respectively. The activation of NF- κ B was assayed by using gel mobility shift assays of nuclear extracts.

RESULTS: Treatment of mice with STZ resulted in hyperglycemia and hypoinsulinemia, which was further evidenced by immunohistochemical staining of islets. However, the diabetogenic effects of STZ were completely prevented when mice were pretreated with CCE. The inhibitory effect of CCE on STZ-induced hyperglycemia was mediated through the suppression of iNOS expression. In rat insulinoma RINm5F cells,

CCE completely protected against interleukin-1 β and interferon- γ -mediated cytotoxicity. Moreover, RINm5F cells incubated with CCE showed significant reductions in interleukin-1 β and interferon- γ -induced nitric oxide production and in iNOS mRNA and protein expression, and these findings correlated well with *in vivo* observations.

CONCLUSION: The molecular mechanism by which CCE inhibits iNOS gene expression appears to involve the inhibition of NF- κ B activation. These results reveal the possible therapeutic value of CCE for the prevention of diabetes mellitus progression.

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Key words: *Cortex cinnamomi*; Diabetes; Streptozotocin; Cytokine; NF- κ B

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INTRODUCTION

Insulin-dependent diabetes mellitus (IDDM) is an autoimmune disease that results from the selective destruction of pancreatic β -cells^[1]. At earlier stages of the disease, histological findings show features of insulinitis characterized by the infiltration of immune cells, such as T lymphocytes, macrophages, and natural killer cells into pancreatic islets. These cells produce and release various cytokines which act as humoral mediators of the immunologic process. Thus, cytokines such as interleukin-1 β (IL-1 β), tumor necrosis factor- α , and interferon- γ (IFN- γ) have been implicated as key effector molecules in β -cell function and viability^[2,3].

Alloxan and streptozotocin (STZ) are two commonly used diabetogenic agents. These structurally diverse compounds have a long history of use in diabetes research and are known to be specifically toxic to pancreatic β -cells. Alloxan is thought to produce oxygen free radicals, whereas STZ releases nitric oxide (NO) and oxygen free radicals

during its metabolism^[4-8].

The biochemical mechanisms that mediate the detrimental effects of cytokines and streptozotocin remain elusive. Recent studies suggest that oxygen free radicals or NO mediates the deleterious effects of cytokine or streptozotocin on β -cell dysfunction and destruction^[9,10]. NO is produced by the oxidation of L-arginine to L-citrulline by nitric oxide synthase (NOS)^[11], and excess NO generated in cells may inhibit mitochondrial metabolism, protein modification, and DNA cleavage, any one of which could lead to insulin secretion impairment and β -cell death^[9,12,13].

The induction of iNOS is regulated by transcription factors that bind to specific sites in the promoter of the iNOS gene. It is known that the activation of the transcription factor NF- κ B, which can be induced by various stimuli, e.g., IL-1 β , INF- γ , bacterial lipopolysaccharide and streptozotocin, is essentially required for iNOS expression^[14,15]. NF- κ B is initially located in the cytoplasm as an inactive form complexed with I κ B, an NF- κ B inhibitory factor. Various inducers cause this dissociation of this complex presumably *via* the phosphorylation of I κ B, and allow NF- κ B to be released from the complex. NF- κ B then translocates to the nucleus, where it interacts with its DNA recognition sites to mediate gene transcription^[16]. In light of the possible role of NO in the pathogenesis of autoimmune diabetes, we^[17,18] and others^[19-21] have previously shown that NF- κ B-dependent NO production plays a key role in the dysfunction and destruction of β -cells.

Cortex cinnamomi (CC) is the name given to the bark of *Cinnamomum cassia* Presl, which belongs to the *Lauraceae* family, and is commonly used in traditional Chinese medicine for treating dyspepsia, gastritis, blood circulation disturbances, and inflammatory disease. Moreover, it has been reported that CC extract (CCE) has anti-inflammatory activity and an anti-thrombotic effect^[22,23]. In the present study, we observed the preventive effects of CCE on cytokine- and STZ-induced pancreatic β -cell damage both *in vitro* and *in vivo*. CCE was found to prevent NF- κ B activation, iNOS mRNA and protein expression, and subsequent NO production, and thus protected RINm5F cells and pancreas from cytokine and STZ induced damage.

MATERIALS AND METHODS

Animals

Specific pathogen-free female ICR mice were purchased from the Korean Research Institute of Chemical Technology (Daejeon, Korea) and housed at our animal facility for one week before use. All mice used were 5-6 wk old, and were kept under specific pathogen-free conditions with free access to a standard commercial diet.

To induce diabetes, mice were injected with STZ *via* a tail vein (100 mg STZ/kg body weight). STZ was dissolved in 0.1 mol/L sodium citrate buffer (pH 4.0) and injected within 5 min. To determine the effects of CCE, mice were divided into the following groups; 1) the non-treated control group, 2) the STZ group, 3) the CCE (100 mg/kg) + STZ group, 4) the CCE (250 mg/kg) + STZ group, and the 5) CCE (500 mg/kg) + STZ group ($n = 7$, each group). Mice were administered CCE twice daily for 7 d by oral gavage starting 1 wk before the STZ injection. The day of

injection was defined as d 1. Control group animals were administered citrate buffer alone, i.e., they were not treated with STZ or CCE. All experimental procedures were approved by the Institutional Animal Care and Use Committee at Wonkwang University, Oriental Medical School. On the day of sacrifice, mice were decapitated without anesthesia and trunk blood was collected into prechilled tubes containing 1 mg/mL of EDTA for insulin and glucose determinations.

Cell culture

RINm5F is an insulinoma cell line that was derived from a rat islet cell tumor^[24]. Cells were purchased from the American Type Culture Collection and grown at 37°C in a humidified 50 mL/L CO₂ atmosphere in RPMI 1640 medium (Gibco BRL) supplemented with 10% fetal bovine serum and 2 mmol/L glutamine, 10000 units/mL of penicillin, 10 mg/mL of streptomycin, and 2.5 μ g/mL of amphotericin B.

Preparation of the Cortex cinnamomi extract

The herb was identified as *Cortex cinnamomi* (CC) by local experts. Voucher samples are preserved for reference in the Herbarium of the Department of Physiology, School of Oriental Medicine, Wonkwang University (ref. Omcphy, 2001-05). For extraction, 200 g of dried CC was added to 1800 mL of water and boiled for 2 h, filtered, and concentrated to 200 mL. The sterile extract (7.03 g) so obtained was stored at -20°C.

MTT cell viability assay

The viabilities of cultured cells were determined by using MTT assays, which involve the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan as described previously^[25]. In brief, after 24 h of incubation, cells (1×10^5 /well) in 96-well plates were washed twice with PBS, and MTT (100 μ g/0.1 mL of PBS) was added to each well. Cells were then incubated at 37°C for 1 h, and DMSO (100 μ L) was added to dissolve the formazan crystals. Absorbance was measured at 570 nm using a model Spectra MAX PLUS (Molecular Devices, Sunnyvale, CA).

Determination of blood glucose and insulin

Blood glucose was determined using a glucose oxidase kit (Asan Pharm. Co., Korea), and plasma insulin was assayed using a standard radioimmunoassay technique (Linco Research Immunoassay, St. Charles, MO).

Nitrite measurement

Biologically produced NO is rapidly oxidized to nitrite and nitrate in aqueous solutions^[26], and thus, nitrite concentrations in cell-free culture supernatant reflect NO production, and may be measured colorimetrically^[27]. Following incubation for 24 h, 100 μ L aliquots of culture supernatants were incubated with 100 μ L of a 1:1 mixture of 10 g/L sulfanilamide in 300 g/L acetic acid and 1 g/L N-(1-naphthyl) ethylenediamine dihydrochloride in 600 g/L acetic acid at room temperature. After 5 min, absorbances were measured at 540 nm using a spectrophotometer. Concentrations were determined using a linear standard curve

drawn using serial dilutions of sodium nitrite in working medium.

Western blot analysis of iNOS

Cells and pancreases were homogenized in 100 μ L of ice-cold lysis buffer (20 mmol/L HEPES, pH 7.2, 10 g/L Triton X-100, 100 g/L glycerol, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μ g/mL leupeptin, 10 μ g/mL aprotinin). Homogenates containing 20 μ g of protein were separated by SDS-PAGE with 100 g/L resolving and 30 g/L acrylamide stacking gel, and transferred to nitrocellulose sheets (Millipore, Bedford, MA). These were then blocked with 20 g/L bovine serum albumin and incubated for 4 h with 1 μ g/mL anti-mouse macrophage iNOS antibody (Transduction Lab, Lexington, KY). iNOS protein expression levels were determined using a Chemi-doc image analyzer (Bio-Rad, Hercules, CA).

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR) for iNOS

Total RNA was isolated from cells and pancreases using TRIzol from Invitrogen (Carlsbad, CA), as instructed by the manufacturer. One microgram of total RNA was transcribed into cDNA in a 20 μ L final volume of reaction buffer (10 mmol/L Tris-HCl, pH 7.4, 50 mmol/L MgCl₂, 1 mmol/L each dNTP) and 2.4 μ mol/L oligo-d(T)16-primer, 1 unit RNase inhibitor, and 2.5 units MuV reverse transcriptase by incubation for 10 min at 21°C and 15 min at 42°C. The reaction was stopped by incubation at 99°C for 5 min. For rat iNOS PCR, aliquots of the synthesized cDNA were added to a 45 μ L PCR mixture containing 10 mmol/L Tris-HCl, 50 mmol/L KCl, 1.5 mmol/L MgCl₂, 0.2 mmol/L of each dNTP, 2 units Taq DNA polymerase, and 0.4 μ mol/L of each PCR primer; iNOS upstream primer, 5'-CCACAATAGTACAATACTACTTGG-3', downstream primer, 5'-ACGAGGTGTTTCAGCGT-GCTCCACG-3'. Amplification was initiated by 3 min of denaturation at 94°C, and amplification was performed using 26 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min and was followed by a single extension step of 5 min at 72°C. β -actin PCR was performed using a 2.5 μ L aliquot of synthesized cDNA using primers at a concentration of 0.15 μ mol/L; β -actin upstream primer, 5'-TGCCCATCTATGAGGGTTACG-3' downstream primer, TAGAAGCATTTCGCGTGCACG-3'. The obtained PCR products were analyzed on ethidium bromide-stained agarose (15 g/L) gels.

Preparation of nuclear extracts

Nuclear extracts were prepared as described previously^[18]. Cells and pancreases were immediately washed twice, scraped into 1.5 mmol/L of ice-cold PBS (pH 7.9), and pelleted at 12000 g for 30 s. Cell pellets were suspended in ice-cold hypotonic lysis buffer (10 mmol/L HEPES, 1.5 mmol/L MgCl₂, 0.2 mmol/L KCl, 0.2 mmol/L phenylmethylsulphonyl fluoride, 0.5 mmol/L dithiothreitol), vortexed for 10 sec, and then centrifuged at 3000 r/min for 5 min. Cells pellets were resuspended with ice-cold hypotonic lysis buffer in the presence of 50 μ L of 100 g/L Nonidet P-40 and then incubated on ice for 25 min. Nuclear fractions were precipitated by centrifugation at 4000

rpm for 15 min, pellets were resuspended in 50-100 μ L of low salt extraction buffer (20 mmol/L HEPES, pH 7.9, 1.5 mmol/L MgCl₂, 250 g/L glycerol, 20 mmol/L KCl, 0.2 mmol/L EDTA, 0.2 mmol/L phenylmethylsulphonyl fluoride, 0.5 mmol/L dithiothreitol) and added dropwise to equal volumes of high salt extraction buffer (20 mmol/L HEPES, pH 7.9, 1.5 mmol/L MgCl₂, 250 g/L glycerol, 80 mmol/L KCl, 0.2 mmol/L EDTA, 0.2 mmol/L phenylmethylsulphonyl fluoride, 0.5 mmol/L dithiothreitol). Mixtures were then incubated with continuous shaking at 4°C for 45 min, centrifuged for 20 min at 12000 g, and nuclear extracts were aliquoted and stored at -80°C. Protein concentrations were determined using the method of Bradford^[28].

Electrophoretic mobility shift assay (EMSA)

The activation of NF- κ B was assayed by using gel mobility shift assays of nuclear extracts from control and treated cells^[18]. As a probe for gel retardation assays, an oligonucleotide containing the κ -chain binding site (κ B, 5'-CCGGT-TAACAGAGGGGGCTTTCAGAG-3') was synthesized and labeled with [α -³²P]dCTP. Labeled oligonucleotides (10 000 cpm), 10 μ g of nuclear extracts, and binding buffer (10 mmol/L Tris-HCl, pH 7.6, 500 mmol/L KCl, 10 mmol/L EDTA, 500 g/L glycerol, 100 ng poly (dI-dC), 1mmol/L DTT) were incubated for 30 min at room temperature in final volume of 20 μ L. Reaction mixtures were analyzed by electrophoresis in 40 g/L polyacrylamide gels in 0.5 \times Tris-borate buffer. Gels were then dried and autoradiographed. Specific binding was controlled by competition with a 50-fold excess of cold κ B oligonucleotide.

Immunohistochemistry

Pancreases removed under anesthesia were immediately placed in fixative (40 g/L formaldehyde solution in 0.1 mol/L PBS) overnight, and washed with 0.1 mol/L PBS. To account for variations between pancreatic regions, tissues were cut into 4-mm blocks, which were then randomly inserted into a cassette and glycol methacrylate (GMA, Technovit 8100) embedded, sectioned (2 μ m), and stained with haematoxylin and eosin (H-E). For immunostaining studies, guinea pig anti-swine insulin antibody (working dilution 1:50; DakoCytomation, Belgium) was incubated with sections for 3 h at 37°C. FITC-conjugated goat anti-guinea pig IgG antibody (working dilution 1:25, Jackson ImmunoResearch Laboratories, Inc, West Grove, PA) was used for fluorescence imaging.

Statistical analysis

Statistical analysis was performed using the Student's *t*-test and ANOVA. *P* values of < 0.05 were considered statistically significant.

RESULTS

CCE has anti-diabetic effect on STZ-induced diabetes in mice

Mice that received 100 mg/kg of STZ became hyperglycemic at 72 h. Their blood glucose levels at 72 h were 16.43 \pm 2.16 mmol/L, a value within the acceptable diabetic

Table 1 Effect of CCE on STZ-induced diabetes in mice

	Control	STZ (100 mg/kg)			
		None	CCE (100 mg/kg)	CCE (250 mg/kg)	CCE (500 mg/kg)
Glucose (mmol/L)	5.88 ± 0.67	16.43 ± 2.16 ^b	8.27 ± 1.44 ^a	7.10 ± 2.55 ^a	6.71 ± 0.89 ^a
Insulin (ng/mL)	0.21 ± 0.04	0.06 ± 0.01 ^b	0.14 ± 0.03 ^a	0.17 ± 0.03 ^a	0.18 ± 0.01 ^a

Values are means ± SE, $n = 7$. ^b $P < 0.01$ vs Control. ^a $P < 0.05$ vs none.

range. In contrast, the mice pretreated with CCE and treated with STZ showed normal blood glucose values (Table 1). Moreover, this glucose lowering effect of CCE was dose-dependent. CCE treatment alone did not affect blood glucose values in mice (data not shown). Animals injected with STZ alone showed significant decreases in plasma insulin levels (0.06 ± 0.01 ng/mL) versus non-treated controls (0.21 ± 0.04 ng/mL). Pretreatment of mice with CCE attenuated the severity of STZ-induced hypoinsulinemia (Table 1). Thus, CCE was protective against STZ-induced diabetes.

CCE protects pancreatic islets from STZ

To elucidate the preventive effect of CCE on STZ-induced diabetes, pancreatic islets were histologically examined. Pancreatic tissues were obtained one week after STZ administration with or without CCE pretreatment and subjected to H-E staining and immunohistochemistry. In diabetic mice not treated with CCE, the most consistent findings in pancreatic sections stained with H-E were degenerative and necrotic changes, and islet shrinkage (Figure 1B). Immunohistochemical staining showed weak insulin reactivity in a few β -cells (Figure 1F). However, diabetic mice pretreated with CCE showed near normal islets by both H-E staining and immunohistochemistry; i.e., round shaped and clearly defined islets with strong insulin positive staining (Figures 1C, 1D, 1G and 1H).

CCE inhibits STZ-induced NF- κ B activation in mice pancreases

To clarify the antidiabetogenic mechanism of CCE, we examined its effect on STZ-induced NF- κ B activation. The findings of this investigation are in accord with those of previous studies, which demonstrated that in mice, STZ treatment results in NF- κ B activation^[10]. Figure 2 shows a representative EMSA radiograph showing the ³²P-DNA/NF- κ B complex in nuclear extracts of pancreas 30 min after STZ injection. In contrast, this complex was not detected in nuclear extracts from CCE-pretreated mice. This result shows that CCE inhibits the translocation of NF- κ B to the nucleus in mice.

CCE inhibits STZ-induced iNOS protein and mRNA expression in mice pancreases

iNOS is a downstream target of NF- κ B activation. Thus, we examined whether CCE modulates the induction of iNOS protein after STZ injection (Figure 3A). STZ in-

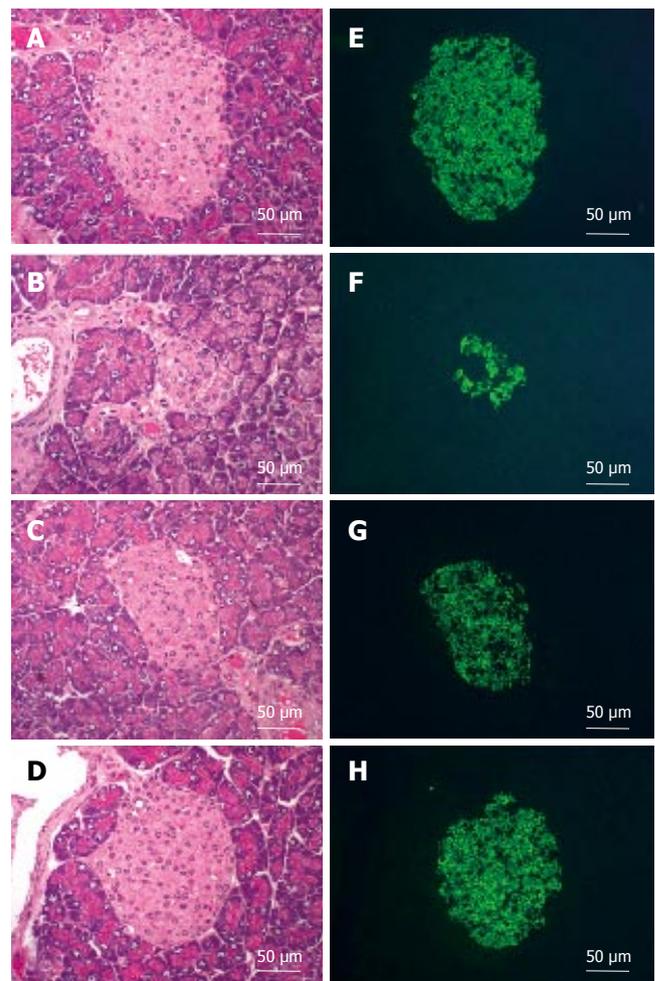


Figure 1 Protection of islets from STZ-induced destruction by CCE. Pancreases were obtained from normal controls (A, E), from STZ-injected (B, F), and from CCE pretreated for 7 d with concentrations of 100 mg/kg per day (C, G) or 250 mg/kg (D, H) subsequently injected with STZ. Islets were labeled with insulin antibody and FITC-labeled anti-guinea pig IgG, and examined by fluorescence microscopy (E-H). The cellular morphologies of these same islets and of adjoining exocrine regions were counterstained with H-E (A-D). Bar = 50 μ m.

jected mice expressed high levels of iNOS protein. However, this STZ-induced upregulation of iNOS protein was totally suppressed by pretreating with CCE. We examined iNOS steady state mRNA levels after STZ injection by RT-PCR (Figure 3B). iNOS mRNA was found to be induced 24 h after STZ injection, whereas no iNOS mRNA was detected in pancreases of CCE-pretreated mice. The results suggest that iNOS, a downstream target of NF- κ B, is induced by STZ injection, and that CCE pretreatment inhibits this induction, possibly by inhibiting NF- κ B.

CCE prevents cytokine-mediated cell death by RINm5F cells

We next investigated the anti-diabetogenic effect of CCE at the cellular level. RINm5F cells, a rat pancreatic β -cell line, were cultured to near confluence, and treated with a high dose combination of cytokines (IL-1 β (2.0 ng/mL) and IFN- γ (100 U/mL)) with/without CCE for 24 h. Percentages of viable cells were detected using MTT assays. As shown in Figure 4, cytokine-treated cells had a viability

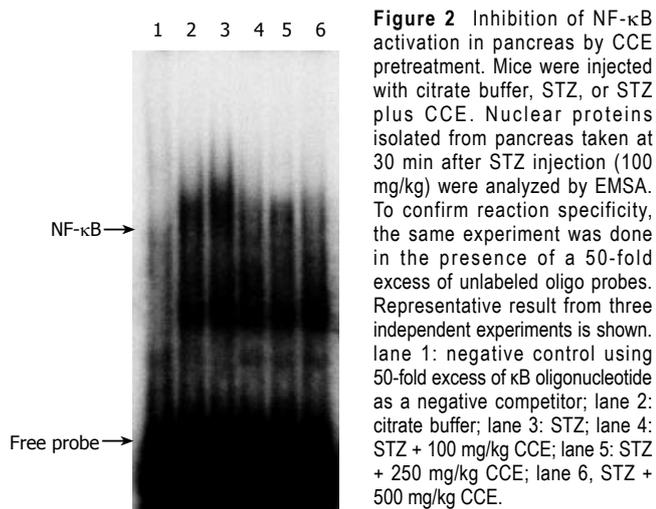


Figure 2 Inhibition of NF-κB activation in pancreas by CCE pretreatment. Mice were injected with citrate buffer, STZ, or STZ plus CCE. Nuclear proteins isolated from pancreas taken at 30 min after STZ injection (100 mg/kg) were analyzed by EMSA. To confirm reaction specificity, the same experiment was done in the presence of a 50-fold excess of unlabeled oligo probes. Representative result from three independent experiments is shown. lane 1: negative control using 50-fold excess of κB oligonucleotide as a negative competitor; lane 2: citrate buffer; lane 3: STZ; lane 4: STZ + 100 mg/kg CCE; lane 5: STZ + 250 mg/kg CCE; lane 6, STZ + 500 mg/kg CCE.

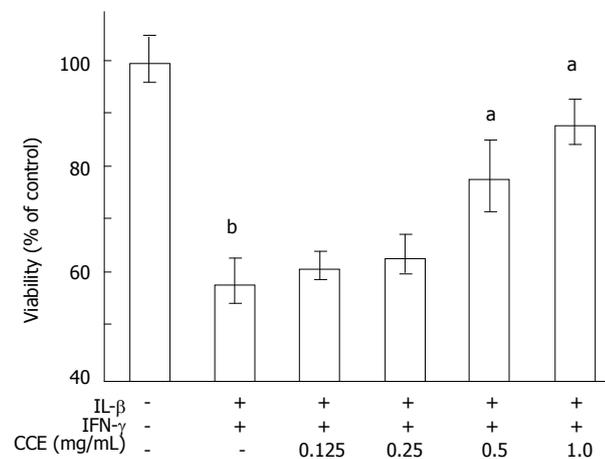


Figure 4 Prevention of cytokine-induced cell death by CCE. RINm5F cells (1×10^5) were incubated with cytokines in the presence or absence of CCE for 24 h. The concentrations of cytokines used were: IL-1β, 2 ng/mL and IFN-γ, 100 U/mL. The percentages of viable cells after these treatments were determined by using MTT colorimetric assays and were calculated versus the A_{570} values of untreated control cells. Value are means \pm SE of four independent experiments. ^b $P < 0.01$ vs control; ^a $P < 0.05$ vs cytokines.

of $58.2\% \pm 4.5\%$, whereas cells pretreated with CCE (0.5 mg/mL) had a viability of $78.2\% \pm 6.8\%$. Moreover, a two-fold higher dose of CCE (1.0 mg/mL) increased cell viability to $88.3 \pm 4.2\%$. CCE alone did not affect the cell viability (data not shown).

NO production was evaluated using the same conditions. Control RINm5F cells generated $19.8 \pm 2.0 \mu\text{mole/L}$ of nitrite, whereas cytokine treated cells generated $51.8 \pm 1.6 \mu\text{mole/L}$ of nitrite in 24 h (Figure 5). On the other hand, RINm5F cells treated with cytokines plus CCE showed concentration dependent reductions in cytokine-mediated nitrite production. Near complete inhibition of nitrite production was observed at a CCE concentration of 1.0 mg/mL. Treatment with CCE alone did not produce nitrite (data not shown).

CCE inhibits cytokine-mediated iNOS expression by inhibiting NF-κB

To investigate the regulatory effect of CCE on NO pro-

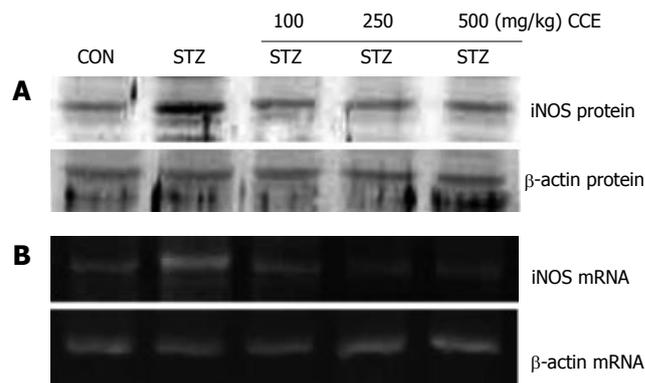


Figure 3 Expression of iNOS protein and mRNA induced by STZ in mice pancreases. Mice were injected with STZ (100 mg/kg) after being pretreated for 7 d with various concentrations of CCE. iNOS protein (A) and mRNA (B) were isolated and detected by Western blotting and RT-PCR, respectively. Representative results from three independent experiments are shown.

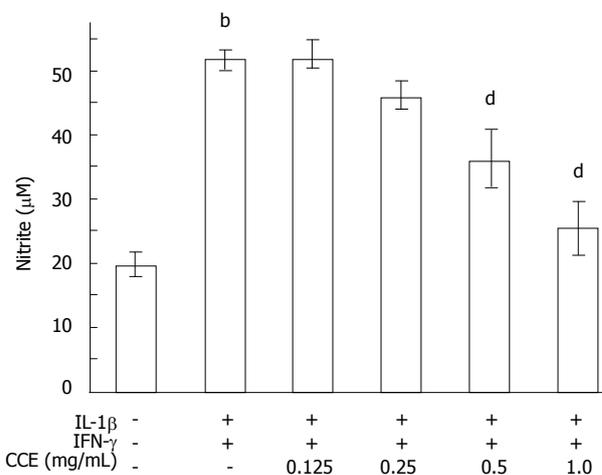


Figure 5 Effect of CCE on cytokine-induced NO production in RINm5F cells. RINm5F cells (5×10^5) were treated with cytokines with or without the indicated concentrations of CCE. The concentrations of cytokines used were same as those in Figure 4. Following 24 h of incubation, nitrite concentrations (indicative of NO synthesis) were measured in cell-free culture supernatants. Results of triplicate samples are expressed as means \pm SE of four independent experiments. ^b $P < 0.01$ vs control; ^d $P < 0.01$ vs cytokines.

duction, we examined the effects of CCE on cytokine-induced iNOS mRNA and protein expressions using RT-PCR and Western blotting, respectively. As shown in Figure 6, the above cytokine combination increased iNOS mRNA and protein levels. However, when CCE was added to the cytokine mixture, iNOS mRNA and protein levels reduced dose-dependently, though 0.125 mg/mL CCE was ineffective, 1.0 mg/mL of CCE completely blocked iNOS mRNA expression.

Because NF-κB has been implicated in the toxicity of STZ (Figure 2), we studied the effect of CCE on the cytokine-stimulated translocation of NF-κB from the cytosol to the nucleus in RINm5F cells. Nuclear extracts from cytokine-stimulated RINm5F cells showed an increase in NF-κB binding activity (Figure 7, lane 2), and this was markedly suppressed by adding CCE, implying that CCE inhibits iNOS expression and NO production by inhibiting NF-κB activation in RINm5F cells. The specificity of the DNA-NF-κB interaction was demonstrated by competitive

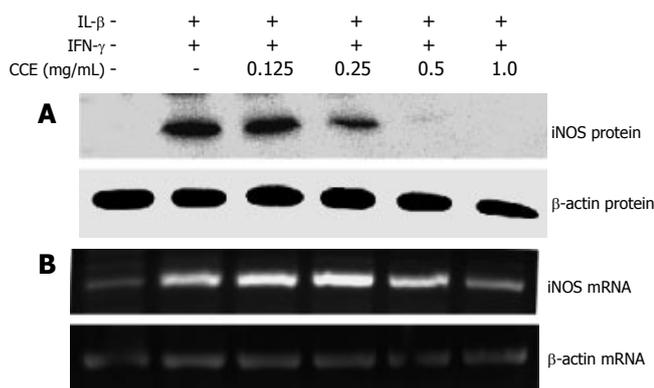


Figure 6 Effect of CCE on cytokine-induced iNOS protein and mRNA expression in RINm5F cells. RINm5F cells (1×10^6) were cultured in 6-well plates and treated with cytokines with or without CCE for 24 h, and then analyzed by Western blotting (A) or RT-PCR (B), as described in "Materials and Methods". The concentrations of the cytokines used were as described in Figure 4. Results are representative of three independent experiments.

assays using a 50-fold excess of unlabeled oligonucleotide (Figure 7, lane 7). These results indicate that the cytokine-induced expression of iNOS protein in RINm5F cells is inhibited by CCE at the transcriptional level.

DISCUSSION

IDDM is an autoimmune disease characterized by the specific destruction of β -cells in the islets of Langerhans^[1]. Cumulative evidence suggest that NF- κ B dependent NO production is a critical component of the destruction and dysfunction of β -cells. NF- κ B activation in β -cells can be triggered by cytokines and by diabetogenic drugs such as STZ and alloxan^[10,14,15]. Therefore, reduced NO production presents a potential means of overcoming β -cell failure.

The use of traditional plant medicines has been practiced for centuries by mankind, and despite a general insufficiency of supportive evidence concerning therapeutic efficacies, herbal medicine usage continues to increase. The present results confirm previous observations that cytokines and STZ induce NF- κ B activation, iNOS expression, NO production, and β -cell dysfunction in both mice pancreases and cultured β -cells, and that CCE abolishes cytokine- and STZ-mediated β -cell damage by blocking NF- κ B translocation to the nucleus. Thereby, these results indicate that CCE can preserve the insulin secreting capacity and viability of β -cells.

In the first series of experiments, we used mice to observe NF- κ B activation by STZ and its prevention by CCE. EMSA studies revealed increased NF- κ B binding activity in pancreatic nuclear extracts derived from STZ-treated hyperglycemic diabetic mice. In contrast, pretreatment with CCE prevented NF- κ B activation and iNOS expression, and this resulted in the maintenance of plasma glucose and insulin levels in the normal range. To further confirm the preventive effect of CCE, histological examinations on pancreatic islets were performed. STZ-treated mice showed marked islet destruction and relative small numbers of insulin-positive β -cells, whereas well-defined islets and strong insulin positive staining were observed in

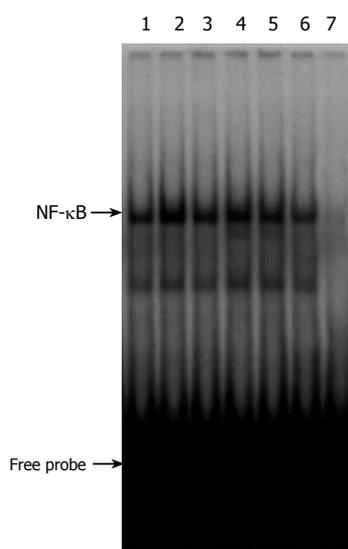


Figure 7 Effect of CCE on the cytokine-induced translocation of NF- κ B from cytosol to the nucleus. RINm5F cells (5×10^6) were treated with cytokines in the presence or absence of CCE. Following 30 min of incubation, nuclear extracts were prepared and NF- κ B activation levels were analyzed by EMSA, as described in "Materials and Methods". The concentrations of cytokines used were the same as in Figure 4. Results representative of three independent experiments are shown. Lane 1: none; lane 2: IL-1 β + IFN- γ ; lane 3: IL-1 β + IFN- γ + 0.125 mg/mL CCE; lane 4: IL-1 β + IFN- γ + 0.25 mg/mL CCE; lane 5, IL-1 β + IFN- γ + 0.5 mg/mL CCE; lane 6, IL-1 β + IFN- γ + 1.0 mg/mL CCE; lane 7: negative control using 50-fold excess of κ B oligonucleotide as a negative competitor.

CCE pre-treated mice. STZ-induced diabetic rodents were previously reported to show increased iNOS activity and expression in islets at a very early stage^[4,5,8]. In this situation, NO generated may react with oxygen free radicals like the superoxide anion, to form highly oxidizing peroxynitrite, which would lead to more aggressive oxidative and nitrosative stress.

Similar effects of CCE were observed in RINm5F cells exposed to cytokines for 24 h. The reduction in NO production induced by CCE in RINm5F cells was associated with a reduction in NF- κ B activation and iNOS mRNA expression. These findings are in line with recent observations that the extract of *A. xanthoides* reduces iNOS expression and protects β -cells in mice and RINm5F cells from alloxan^[17,18]. Future studies are needed to determine the target pathway that leads to the sequestration of NF- κ B in the cytosol.

The generation of oxygen free radicals is another important mechanism of cytokine- and other diabetogenic drug-mediated toxicities in β -cells. Experimental evidence indicates that oxygen free radicals are generated in both cytokine-stimulated^[6,29,30] and STZ-treated β -cells^[31], and that the overexpression of antioxidant enzymes protects β -cells from such insults^[7,32,33]. Furthermore, oxygen free radicals induce NF- κ B activation and iNOS expression in rodent and human β -cells^[34,35], which suggests a role for hydrogen peroxide in the pathway of NF- κ B activation and iNOS expression induced by cytokines. We cannot exclude the possibility that the protective effect of CCE occurs *via* the reduced generation of oxygen free radicals.

In summary, our results indicate that CCE inhibits cytokines- and STZ-induced β -cell damage *in vivo* and *in vitro*. The primary mechanism underlying this effect is the inhibition of iNOS protein expression, which may be mediated at the transcriptional level through the inhibition of NF- κ B activation and iNOS transcription. These findings suggest that CCE is likely to have a beneficial effect when used to prevent or attenuate the progress of diabetes mellitus.

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BASIC RESEARCH

Amplification of D22S283 as a favorable prognostic indicator in liver fluke related cholangiocarcinoma

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Abstract

AIM: To analyze the DNA copy number of target genes *NF2*, *TIMP3*, *ST13*, *TOB2*, *BIK*, and *TP* and the reference microsatellite markers D22S283, D22S423, and D22S274 mapped on 22q12-qter in liver fluke related cholangiocarcinoma (CCA) and define its correlation with clinical parameters.

METHODS: Quantitative real time PCR (qPCR) was used for determining allelic imbalances in 65 liver fluke related CCA tissues. Statistical correlations between allelic imbalances and clinicopathological parameters, i.e. age, sex, tumor stage, histological type, blood vessel invasion, nerve invasion and lymphatic invasion were evaluated by means of the χ^2 test. Cox regression analysis was used for determining patient's survival.

RESULTS: Amplifications of the *TP* (22q13.33), *TOB2* (22q13.2-13.31), D22S283 (22q12.3), *TIMP3* (22q12.3) and *NF2* (22q12.2) were found in 35 (53.8%), 28 (43.1%), 27 (41.5%), 24 (36.9%), and 24 (36.9%), respectively. Losses at the D22S423 (22q13.1-13.2)

and *BIK* (22q13.31) were detected in 26 (40%) and 23 (35.4%), respectively. Significant correlations were observed between lymphatic invasion and allelic losses of *BIK* ($P = 0.025$) and D22S283 ($P = 0.041$). Univariate and multivariate Cox regression analysis revealed D22S283 amplification as an independent predictor of good prognosis ($P = 0.006$, death hazard ratio = 0.411, 95% CI = 0.217-0.779) and blood vessel invasion as an independent poor prognostic factor ($P = 0.042$, death hazard ratio = 1.911, 95% CI = 1.022-3.571) in CCA patients.

CONCLUSION: This study provides evidence for the involvement of gene amplification and deletion on chromosome 22q in liver fluke related CCA. This is the first report of D22S283 amplification as an independent indicator of favorable prognosis in liver fluke related CCA.

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Key words: Liver fluke related cholangiocarcinoma; Chromosome 22q; D22S283; Allelic imbalance; Quantitative real time PCR

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INTRODUCTION

Cholangiocarcinoma (CCA) which arises from bile duct epithelium is a common hepatobiliary malignancy found in Northeastern Thais. Liver fluke (*Opisthorchis viverrini*) infection related to cholangiocarcinogenesis is strongly supported by evidence from both experimental and epidemiological studies^[1,2]. In addition, exogenous and/or endogenous N-nitroso compounds have also been claimed to be responsible^[1,2]. These studies suggest that liver fluke infection causes chronic inflammatory reactions and enhance the susceptibility of bile duct epithelium to carcinogenic chemicals such as N-nitroso compounds leading to genetic and epigenetic damages in the cells. CCA accounts for about 89% of all liver cancer cases

in Khon Kaen with the highest incidence in the world ($97.4/10^5$ in males and $39.0/10^5$ in females)^[3]. Khon Kaen is one of the largest provinces in Northeast Thailand where the liver fluke is highly endemic. Most patients are diagnosed at late stage and difficult to cure successfully because of advanced metastasis at the time of diagnosis. The 3-year survival rates are 33%, 30% and 12% for stage III, stage IVa, and stage IVb, respectively, whereas 5-year survival rates are 0% for all three late stages^[4]. CCA, like other common epithelial cancers is believed to develop through a multistep process. However, the molecular mechanism of carcinogenesis of CCA remain unclear. A series of different genes and chromosomal regions may be deleted or amplified in the tumor genome. Several genes are reported to be involved in CCA, e.g. *p53* and *MDM2*^[5], *p16*^[6], *K-ras*^[6], *bMLH1* and *bMSH2*^[7,8], and *COX-2*^[9,10]. At the chromosomal levels, changes of several chromosomal arms have been reported, e.g. 13q^[11], 6q, 9p and 17p^[12], 17q, 5p, 6q, and 18q^[13].

There are reports of genetic changes on the chromosomal region 22q in other epithelial cancers such as colorectal cancer^[14,15], oral cancer^[16], breast cancer^[17], gastric carcinoma^[18] and ovarian carcinoma^[19], except CCA. Alteration in gene copy number by amplification or deletion is a common mechanism that leads to deregulation of gene expression and finally to neoplastic transformation. Investigation of the prognostic or predictive significances of these genetic alterations requires a reliable and sensitive method for the measurement of gene copy number in clinical tumor samples. Quantitative real time PCR is increasingly used to quantify copy numbers of nucleic acids in clinical applications^[20]. The measurement of gene copy number by qPCR has frequently been reported for human tumors including stomach cancer^[21], neuroblastoma^[22], and oligodendroglioma^[23]. Our comparative genomic hybridization data in CCA showed copy number alteration at 22q13 at 21%. Taken together, this study aimed to investigate the allelic imbalance of the chromosomal region 22q12-qter by means of SYBR Green I-based qPCR assay and define its candidate genes which may be involved in carcinogenesis and pathogenesis of liver fluke related CCA. We also correlated our findings with clinical parameters including patient survival.

MATERIALS AND METHODS

Samples and DNA extraction

Sixty-five samples were obtained from liver fluke related CCA patients undergoing surgery at Srinagarind Hospital, Khon Kaen University, Khon Kaen, Thailand. The patients' data and survival time are shown in Table 1. Informed consent was obtained from each patient prior to sampling under the guideline approved by the Ethical Committee of Khon Kaen University. Tumor tissues containing 70% or more tumor cells were prepared for DNA extraction following standard method. Peripheral blood samples collected from 50 healthy blood donors were used to extract DNA which was then pooled to yield 5 pooled DNA samples (10 cases each) for normal DNA copy number determination. DNA extracted from normal

placenta was used for setting a standard curve.

Polymorphic markers and gene specific primers

Six target genes and 3 reference microsatellite markers spanning from centromere to telomere of the chromosomal region 22q12-qter were selected for DNA copy number quantification. The genes were *NF2* (Neurofibromin 2), *TIMP3* (Tissue Inhibitor of Metalloproteinase 3), D22S283, D22S423, *ST13* (Suppression of Tumorigenicity 13), *TOB2* (Transducer of ERBB2, 2), *BIK* (BCL2-interacting Killer), D22S274, and *TP* (Thymidine Phosphorylase). The sequence and location of each locus are shown in Table 2.

Quantitative real time PCR system using a standard curve method

Allelic imbalance on the chromosomal region 22q12-qter was analyzed by SYBR Green I based qPCR using Rotor-Gene 2000 Real Time Amplification System (Corbett Research, Australia). Since SYBR Green I dye is a nonspecific dsDNA intercalating dye, we made the reaction specific by analyzing the annealing and signal acquisition temperatures for each primer. A relative standard curve was constructed for each locus using a 2-fold serial dilution of human placental DNA to get 4 different concentrations. Placental DNA with known concentration was used as a control to validate experimental precision. A relative DNA copy number was calculated for each sample as a ratio of DNA copy number for target locus and the average DNA copy number for 2 reference loci GAPDH and β -actin housekeeping genes. Relative DNA copy numbers of 9 tested loci in 5 pooled normal leukocyte DNA ($n = 45$) were calculated for normal reference range. The relative DNA copy number in the sample was interpreted as loss or gain when the ratio was less than mean - 2SD or more than mean + 2SD of normal reference range, respectively.

Quantitative PCR was conducted in triplicate in a 20 μ L reaction volume containing 67 mmol/L Tris Base, 16.6 mmol/L ammonium sulfate in a 10 mL/L Tween 20, 3 mmol/L MgCl₂, 5 pmol each primer, 100 μ mol/L each deoxynucleoside triphosphate (dNTP), 1X SYBR Green I and 1 unit Taq polymerase with different concentration of genomic DNA. Each locus was amplified using optimal conditions. The thermal cycling conditions comprised 94°C for 300 s, and 40 cycles of 94°C for 15 s, 46°C -58°C for 20 s and 72°C for 15 s and hold at 72°C for 600 s. To assure the reproducibility and accuracy of qPCR, experiments were repeated when a coefficient of variation for triplicate samples was higher than 10% or a PCR efficiency lower than 0.85 was observed.

Statistical analysis

The association between allelic imbalance and clinical features was analyzed by χ^2 test using SPSS statistical software version 10.0 for Windows (SPSS Inc, Chicago, Ill). Survival curves were analyzed by Kaplan-Meier and the significant difference confirmed by Log rank test. Univariate and multivariate Cox regression models were also used for survival analysis. $P < 0.05$ was defined as significance.

Table 1 Patients' data including survival time

No.	Age(yr)	Sex	Staging	Histological grading	Invasion			Survival time (wk)
					Vessel	Nerve	Lymph	
1	61	Female	IVa	Adenosquamous	Yes	No	Yes	16.85
2	50	Male	IVb	Well differentiated	Yes	Yes	Yes	27.28
3	56	Male	IVb	Poorly differentiated	Yes	No	Yes	4.85
4	54	Male	IVa	Well differentiated	Yes	No	No	56.57
5	46	Male	IVb	Adenosquamous	Yes	No	Yes	7.71
6	53	Male	IVa	Well differentiated	Yes	No	Yes	42.14
7	64	Male	IVa	Poorly differentiated	Yes	No	Yes	7.28
8	66	Male	IVb	Poorly differentiated	Yes	No	Yes	7.00
9	72	Male	IVb	Unclassified	No	No	Yes	20.14
10	64	Female	IVb	Unclassified	Yes	Yes	Yes	7.14
11	49	Male	IVb	Well differentiated	Yes	No	No	15.57
12	53	Male	III	Poorly differentiated	Yes	No	Yes	242.85
13	53	Female	IVa	Well differentiated	Yes	No	No	2.00
14	62	Male	IVb	Adenosquamous	Yes	Yes	Yes	3.00
15	56	Male	II	Poorly differentiated	Yes	No	Yes	30.71
16	55	Male	IVb	Moderately differentiated	Yes	No	Yes	72.57
17	68	Male	IVb	Moderately differentiated	Yes	Yes	Yes	32.00
18	72	Female	IVb	Poorly differentiated	Yes	No	No	18.57
19	65	Female	IVb	Well differentiated	Yes	Yes	Yes	20.85
20	52	Male	IVa	Moderately differentiated	Yes	Yes	Yes	36.71
21	53	Male	IVb	Unclassified	Yes	No	No	27.14
22	66	Male	IVb	Moderately differentiated	Yes	Yes	Yes	10.42
23	49	Male	IVb	Well differentiated	No	Yes	Yes	67.71
24	56	Male	IVb	Adenosquamous	Yes	No	Yes	10.00
25	41	Male	IVb	Moderately differentiated	No	Yes	No	47.14
26	67	Female	IVb	Poorly differentiated	No	No	Yes	118.28
27	63	Male	IVa	Unclassified	Yes	Yes	Yes	73.85
28	39	Female	IVa	Well differentiated	Yes	Yes	Yes	30.42
29	44	Male	IVb	Unclassified	Yes	Yes	Yes	18.71
30	60	Female	IVb	Poorly differentiated	Yes	No	Yes	75.71
31	40	Male	III	Poorly differentiated	Yes	No	Yes	26.57
32	62	Male	IVb	Unclassified	No	No	Yes	40.00
33	43	Female	IVb	Moderately differentiated	No	Yes	Yes	29.42
34	70	Male	IVa	Unclassified	Yes	No	Yes	79.42
35	63	Female	III	Unclassified	No	No	No	80.71
36	61	Male	IVb	Moderately differentiated	No	Yes	Yes	77.28
37	48	Male	III	Unclassified	No	No	No	94.00
38	55	Male	IVb	Moderately differentiated	Yes	Yes	Yes	12.14
39	50	Female	IVb	Moderately differentiated	No	Yes	Yes	40.14
40	39	Male	IVb	Adenosquamous	Yes	No	Yes	9.14
41	58	Male	IVb	Well differentiated	Yes	Yes	Yes	70.42
42	40	Female	III	Moderately differentiated	No	Yes	No	17.42
43	36	Female	IVb	Well differentiated	No	Yes	Yes	35.14
44	75	Male	IVb	Poorly differentiated	Yes	No	Yes	22.14
45	40	Male	III	Well differentiated	No	Yes	Yes	3.42
46	52	Male	IVa	Adenosquamous	No	Yes	Yes	49.00
47	49	Male	IVb	Moderately differentiated	No	Yes	Yes	27.42
48	63	Male	IVb	Well differentiated	Yes	Yes	Yes	18.28
49	50	Male	IVb	Adenosquamous	Yes	Yes	Yes	35.00
50	56	Male	III	Poorly differentiated	Yes	No	Yes	41.14
51	61	Male	IVb	Poorly differentiated	Yes	No	No	3.00
52	58	Male	IVb	Well differentiated	Yes	No	Yes	13.00
53	55	Female	IVb	Well differentiated	Yes	Yes	Yes	67.85
54	55	Female	IVb	Poorly differentiated	No	Yes	Yes	40.85
55	50	Male	nd	Well differentiated	Yes	No	Yes	10.71
56	38	Male	IVb	Well differentiated	Yes	Yes	Yes	27.85
57	46	Female	nd	Well differentiated	Yes	Yes	Yes	9.71
58	43	Male	IVb	Well differentiated	Yes	No	Yes	65.00
59	54	Male	IVa	Well differentiated	No	No	No	9.14
60	42	Male	IVb	Moderately differentiated	No	Yes	Yes	7.14

61	34	Male	IVa	Well differentiated	Yes	Yes	Yes	9.00
62	61	Female	IVa	Unclassified	No	No	Yes	89.14
63	62	Female	IVb	Poorly differentiated	Yes	Yes	Yes	45.28
64	51	Male	IVa	Well differentiated	Yes	Yes	Yes	55.42
65	46	Male	IVa	Poorly differentiated	Yes	Yes	Yes	18.71

Table 2 Sequences of markers located at chromosomal region 22q12-qter including two housekeeping genes

Gene/ marker	Band	Primer sequence (5'-3')
D22S283	22q12.3	5' ACC AAC CAG CAT CAT CAT 3' 5' AGC TCG GGA CTT TCT GAG 3'
D22S423	22q13.1-13.2	5' TGC AAA CTC AGC CTG GA 3' 5' ACC AAC TGA CTC GTT TAG GTC AT 3'
D22S274	22q13.3	5' GTC CAG GAG GTT GAT GC 3' 5' AGT GCC CAT TTC TCA AAA TA 3'
NF2	22q12.2	5' AAG AGC AAG CAT CTG CAG GA 3' 5' TGG TAT TGT GCT TGC TGC TG 3'
TIMP3	22q12.3	5' TGT CTC TGG ACC GAC ATG CT 3' 5' TGG CGC TCA GGG ATC TGT G 3'
ST13	22q13.2	5' GTT ACA CTA TTT AAG AGC TGA AT 3' 5' GGT CTT CTA CTT AGA AAA ACC TA 3'
TOB2	22q13.2-q13.31	5' GAA GAC ACC CCT TTG TGG AA 3' 5' TCT GIG GTC TTG GGT GCT C 3'
BIK	22q13.31	5' ATG ACC ACT GCC CTG GAG 3' 5' CTA AAC ACA GGC CAC AGT TAA CC 3'
TP	22q13.33	5' GGG GCT CAA GTC GCG AGG 3' 5' CCT GCG GGG ATG CCT GAC 3'
β -actin (Reference)	7p15-p12	5' TCA CCC ACA CTG TGC CCA TCT ACG A 3' 5' CAG CGG AAC CGC TCA TTG CCA ATG G 3'
GAPDH (Reference)	12p13	5' ACA GTC CAT GCC ATC ACT GCC 3' 5' GCC TGC TTC ACC ACC TTC TTG 3'

RESULTS

Allelic imbalance of 9 target loci on chromosome 22q12-qter

Sixty-five samples from liver fluke related CCA patients were investigated for aberrations in DNA copy number of 9 target genes on 22q12-qter. The normal reference range statistically calculated from 5 pooled normal leukocyte DNA was 0.82-1.33, which was derived from 1.08 ± 0.13 (mean \pm 2SD; $n = 45$). Amplification frequencies higher than 30% were observed in TP (53.8%), TOB2 (43.1%), D22S283 (41.5 %), TIMP3 (36.9%), and NF2 (36.9%). Loss frequencies more than 30% were detected in D22S423 (40%) and BIK (35.4%). Allelic imbalance of each locus is shown in Figure 1. Fine mapping of chromosomal region 22q12-qter from centromeric (NF2) to telomeric ends (TP) in CCA is shown in Figure 2. The regions of common amplification were observed at TP, TOB2 and the 6.6 cM region between NF2 and D22S283. The regions of common loss were D22S423 and BIK.

Correlation of clinicopathological features and allelic imbalance on chromosome 22q12-qter

Associations between allelic imbalances of 9 target loci and clinicopathological parameters, i.e. age, sex, tumor stage, histological type, blood vessel invasion, nerve

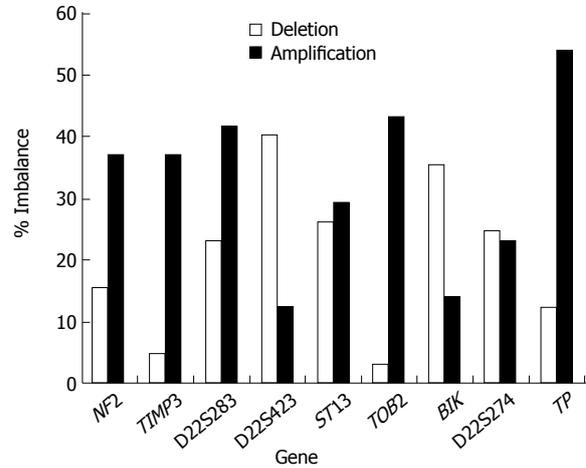


Figure 1 Percentages of deletion and amplification in each locus.

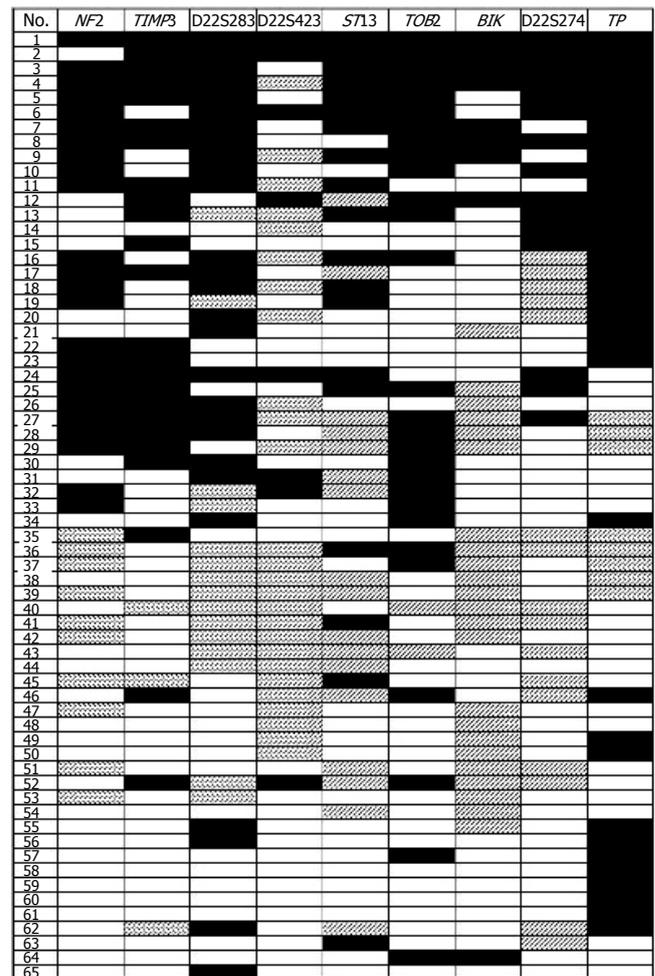


Figure 2 Fine mapping of chromosomal region 22q12-qter in CCA from centromeric (NF2) to telomeric (TP) ends. The regions of common amplification are TP, TOB2, and between NF2 and D22S283. The common deleted regions are D22S423 and BIK. □ no change of DNA copy number, ▨ deletion, and ■ amplification.

Table 3 Cox regression analysis using univariate and multivariate models in CCA patients

Variables	Categories	Univariate analysis		Multivariate analysis	
		P-value	HR (95% CI)	P-value	HR (95% CI)
Stage	IVa + IVb vs II + III	NS	1.560 (0.660-3.690)		
Histology	Poorly vs Unclassified	NS	1.152 (0.507-2.619)		
	Moderate vs Unclassified	NS	0.994 (0.473-2.087)		
	Well diff vs Unclassified	NS	0.929 (0.429-2.013)		
Vessel invasion	Yes vs No	0.021	2.080 (1.120-3.877)	0.042	1.911 (1.022-3.571)
Nerve invasion	Yes vs No	NS	1.328 (0.770-2.293)		
Lymphatic invasion	Yes vs No	NS	1.591 (0.716-3.536)		
TP	Deletion vs Normal	NS	0.791 (0.314-1.997)		
	Amplification vs Normal	NS	1.034 (0.576-1.857)		
D22S274	Deletion vs Normal	NS	1.186 (0.625-2.250)		
	Amplification vs Normal	NS	0.869 (0.439-1.718)		
BIK	Deletion vs Normal	NS	1.271 (0.704-2.294)		
	Amplification vs Normal	NS	1.714 (0.771-3.810)		
TOB2	Deletion vs Normal	NS	1.176 (0.277-4.995)		
	Amplification vs Normal	NS	0.945 (0.545-1.641)		
ST13	Deletion vs Normal	NS	0.913 (0.466-1.788)		
	Amplification vs Normal	NS	0.938 (0.500-1.761)		
D22S423	Deletion vs Normal	NS	1.183 (0.667-2.097)		
	Amplification vs Normal	NS	0.896 (0.366-2.192)		
D22S283	Deletion vs Normal	NS	0.793 (0.397-1.585)		
	Amplification vs Normal	0.003	0.384 (0.202-0.728)	0.006	0.411 (0.217-0.779)
TIMP3	Deletion vs Normal	NS	1.758 (0.527-5.864)		
	Amplification vs Normal	NS	0.736 (0.418-1.296)		
NF2	Deletion vs Normal	NS	0.929 (0.420-2.055)		
	Amplification vs Normal	NS	0.621 (0.344-1.122)		

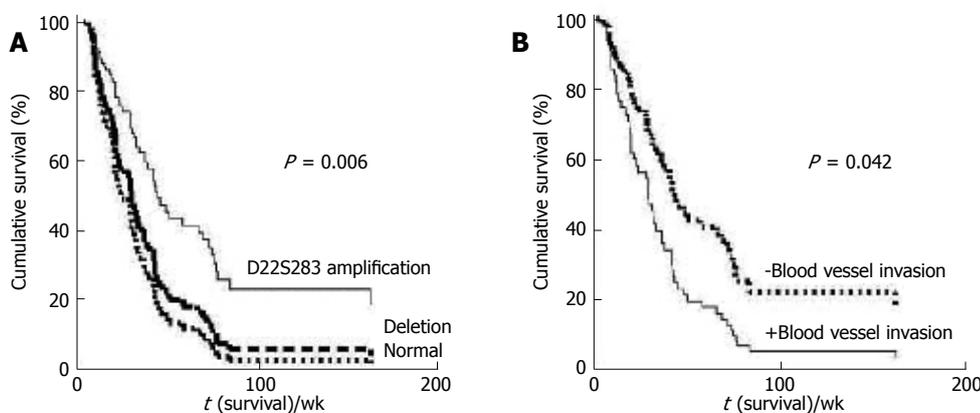


Figure 3 Survival curve by multivariate Cox regression analysis. **A:** Multivariate Cox regression analysis revealed D22S283 copy number amplification as an indicator of favorable prognosis; **B:** blood vessel invasion showed poor prognosis in liver fluke related CCA patients.

invasion and lymphatic invasion were evaluated by means of the χ^2 test. The mean age of the 65 patients was 54 years. Lymphatic invasion was observed in patients with deletion at D22S283 ($P = 0.041$) and *BIK* ($P = 0.025$). Using Kaplan-Meier survival analysis, the median survival time was 30.42 weeks (SE = 4.29, 95% CI = 22.00-38.84), while the 3-yr and 5-yr survival rate were 9.2% and 1.5%, respectively. Cox regression analysis by univariate model showed poor outcome for patients with blood vessel invasion ($P = 0.021$, death hazard ratio = 2.080, 95% CI = 1.120-3.877) but improved survival with D22S283 amplification ($P = 0.003$, death hazard ratio = 0.384, 95% CI = 0.202-0.728) (Table 3). Parameters with $P < 0.1$ in the univariate analysis were included in the multivariate model of Cox regression analysis for survival. The multivariate analysis revealed the significance of D22S283 amplification

as an independent predictor of better prognosis compared with those without ($P = 0.006$, death hazard ratio = 0.411, 95% CI = 0.217-0.779), whereas blood vessel invasion was revealed as an independent poorer prognostic factor in CCA patients ($P = 0.042$, death hazard ratio = 1.911, 95% CI = 1.022-3.571) as shown in Table 3. Survival prediction by multivariate Cox regression analysis of D22S283 and blood vessel invasion is shown in Figure 3.

DISCUSSION

Gene deletion and amplification are common events in tumorigenesis and progression. In this study, we examined the allelic imbalance of chromosomal region 22q12-qter by SYBR Green I-based qPCR assay and determined its association with clinicopathological parameters and

survival in 65 CCA patients. *TP* showed the highest frequency of genomic DNA amplification (53.8%) (Figure 1), suggesting its important role in the development of CCA. *TP* gene located at 22q13.33 is an angiogenic factor, which promotes angiogenesis *in vivo* and stimulates the *in vitro* growth of a variety of endothelial cells. *TP* also has an enzymatic activity involved in pyrimidine metabolism. It catalyzes the phosphorolysis of thymidine to thymine and deoxyribose-1-phosphate and has a pro-angiogenic effect for which deoxyribose-1-phosphate may be responsible^[24-26]. Since gene amplification is reported to play an important role in the initiation and progression of tumors^[27], *TP* amplification and overexpression have been reported in many solid tumors with invasion and metastasis including CCA^[28-30]. These observations indicate that the incidence of amplification and overexpression of *TP* is high in cancer and associated with carcinogenesis. Although we could not find any correlation between clinicopathological parameters and *TP* imbalance or impact of *TP* gene copy number on patient survival when treated with 5-fluorouracil (5-FU)-based chemotherapy (data not shown), its high amplification may be worth further investigation into its involvement in chemotherapeutic activity because normal *TP* protein activity is required for the activation of the cytotoxic activities of anti-tumor drug 5-FU. 5-FU-based chemotherapy is given to patients with advanced cancer and as an adjuvant treatment. 5-FU is a fluorinated pyrimidine that is metabolized intracellularly to its active form, fluorodeoxyuridine monophosphate (FdUMP), by *TP* to inhibit DNA synthesis. Our further studies will investigate whether *TP* expression also associates with chemosensitivity in CCA.

Microsatellite marker D22S423 was the most frequent locus loss (40%) among 9 loci, suggesting that this region may harbor a putative tumor suppressor gene (TSG), which plays a role in the development of CCA. LOH frequency at D22S423 was reported in 26.8% of informative cases in sporadic colorectal cancer^[15]. Putative TSG located 1 cM distal to D22S423 are *ST13* and *EP300*, however, the presence of unknown TSG located within this region cannot be excluded. *ST13* encodes an adaptor protein that mediates the association of the heat shock proteins *HSP70* and *HSP90*. The expression of *ST13* is reported to be downregulated in colorectal carcinoma tissue compared with that in adjacent normal tissue, suggesting that *ST13* is a candidate TSG^[31]. Function and role of *ST13* in tumor development is still unclarified. Further investigation into *ST13* expression and its roles in carcinogenesis of CCA is needed.

D22S283 was deleted in 15 cases, which all had lymphatic invasion, suggesting the deletion of unknown TSG, which functions on inhibiting invasion. D22S283 showed amplification in 27 out of 65 cases, indicating the existence of putative oncogene (s) at this location, for example, *RAB* member of *RAS* oncogene family, RNA binding motif protein 9, and eukaryotic translation initiation factor 3. On the other hand, some TSG at this location may amplify, leading to high expression which may result in good prognosis of patients with D22S283 amplification (Figure 3). Other putative TSG around D22S283 that play a role in apoptosis are caspase recruitment domain family,

member 10 and phospholipase A2, group VI.

All 23 cases of *BIK* gene deletion were associated with lymphatic invasion. Many reports suggest that *BCL2-interacting killer* (apoptosis-inducing) functions as a proapoptotic protein, which enhances programmed cell death. Germain *et al.*^[32] identified *BIK* as an initiator of cytochrome C released from mitochondria operating from a location at the endoplasmic reticulum and activated caspases. Thus, role of *BIK* in pathogenesis and carcinogenesis of CCA needs further investigation. *NF2* gene located at 22q12.2 produces the Merlin protein. It is thought to act as a tumor suppressor protein, however, the mechanism remains obscure. Xiao *et al.*^[33] have shown that merlin inhibits tumor cell proliferation and arrests cells at G₁ phase, concomitant with decreased expression of cyclin D1, inhibition of CDK4 activity, and dephosphorylation of pRB. They suggested a unifying mechanism by which merlin inactivation might contribute to the overgrowth seen in both noninvasive and malignant tumors. DNA amplification of *NF2* was found in 36.9 % of our CCA cases, hence, expression of *NF2* may lead to significant reduction of proliferation and G₀/G₁ arrest in CCA cells.

The development of tumor is a multistep process that requires accumulated mutations or alterations of both oncogenes and tumor suppressor genes. It is generally believed that mutations in 5 to 10 genes are required for the development of a tumor. Thus, *TP*, *BIK*, *NF2*, and candidate genes on D22S423 and D22S283 are likely to play significant roles in carcinogenesis of liver fluke related CCA by this study. In addition, to our knowledge, we first propose that the D22S283 amplification acts as an independent indicator of favorable prognosis in liver fluke related CCA.

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Influence of zinc sulfate intake on acute ethanol-induced liver injury in rats

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Abstract

AIM: To investigate the role of metallothionein and proliferating cell nuclear antigen (PCNA) on the morphological and biochemical effects of zinc sulfate in ethanol-induced liver injury.

METHODS: Wistar albino rats were divided into four groups. Group I; intact rats, group II; control rats given only zinc, group III; animals given absolute ethanol, group IV; rats given zinc and absolute ethanol. Ethanol-induced injury was produced by the 1 mL of absolute ethanol, administered by gavage technique to each rat. Animals received 100 mg/kg per day zinc sulfate for 3 d 2 h prior to the administration of absolute ethanol.

RESULTS: Increases in metallothionein immunoreactivity in control rats given only zinc and rats given zinc and ethanol were observed. PCNA immunohistochemistry showed that the number of PCNA-positive hepatocytes was increased significantly in the livers of rats administered ethanol + zinc sulfate. Acute ethanol exposure caused degenerative morphological changes in the liver. Blood glutathione levels decreased, serum alkaline phosphatase and aspartate transaminase activities increased in the ethanol group when compared to the control group. Liver glutathione levels were reduced, but lipid peroxidation increased in the livers of the group administered ethanol as compared to the other groups. Administration of zinc sulfate in the ethanol group caused a significant decrease in degenerative changes, lipid peroxidation, and alkaline phosphatase and aspartate transaminase activities, but an increase in

liver glutathione.

CONCLUSION: Zinc sulfate has a protective effect on ethanol-induced liver injury. In addition, cell proliferation may be related to the increase in metallothionein immunoreactivity in the livers of rats administered ethanol + zinc sulfate.

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Key words: Liver; Ethanol; Zinc sulfate; Metallothionein; Proliferating cell nuclear antigen; Rat

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INTRODUCTION

The liver is the main site of ethanol biotransformation and it plays a key role in zinc metabolism^[1,2]. Acute ethanol exposure causes liver injury in experimental animals. The mechanisms of ethanol-induced liver injury are not fully elucidated^[3,4]. Metallothionein is an intracellular protein capable of binding metals and can scavenge reactive oxygen species^[5,6]. The mechanism of action for metallothionein is also unknown. Although all tissues are able to synthesize metallothioneins, the main place of synthesis is in the liver^[7]. Ethanol is a potent inducer of liver metallothionein^[8-12]. Proliferating cell nuclear antigen (PCNA) is an essential protein in both DNA replication, DNA repair and possibly cell-cycle control^[13]. Zinc is an essential nutrient that is required in humans and animals for many physiological functions, including antioxidant functions. Zinc has been shown to be essential for the structure and function of a large number of macromolecules and is also essential for over 300 enzymatic reactions^[14]. Zinc is an antioxidative element and it probably mediates the protective action of metallothionein^[15]. Changes in the metabolism of some elements such as zinc can lead to disorders in the antioxidant defense system of liver^[16-17]. It has been shown that zinc plays an important role in the maintenance of glutathione^[18]. Zinc deficiency is important in liver damage, although it is unclear whether zinc supplementation has a place in the treatment of ethanol-induced liver injury^[19,20].

Although some studies have addressed the relationship between zinc-metallothionein and ethanol toxicity, the results obtained from these studies are in conflict^[4,21,22]. The aim of this study was to examine the role of metallothionein and PCNA, and the effects of zinc sulfate on ethanol-induced liver injury morphologically and biochemically.

MATERIALS AND METHODS

Experimental design and treatment of animals

In this study, 2.5-3 mo-old male Wistar albino rats ($n = 41$) from DETAM (Istanbul University, Centre for Experimental Medical Research and Application) were used. The experiments were reviewed and approved by the Local Institute's Animal Care and Use Committees. The animals were fed with pellet chow and tap water *ad libitum*. The animals were randomly divided into four groups. Group I ($n = 8$) was intact control animals. The control rats of group II ($n = 8$) were treated only with 100 mg/kg per day zinc sulfate ($ZnSO_4 \cdot 7H_2O$) (Merck) for 3 d, by gavage technique. The animals of group III ($n = 14$) received 1 ml of absolute ethanol once, by the same method. The animals of group IV ($n = 11$) were treated with zinc sulfate and absolute ethanol at the same dose and time. After 2 h from the time when the last dose of zinc sulfate was given, acute ethanol toxicity on the liver of rats was produced by administration of absolute ethanol. The animals were sacrificed by ether 2 h after treatment with absolute ethanol.

Morphological study

On the 3rd day of the experiment, all of the animals were fasted overnight and sacrificed under ether anesthesia. Liver samples were taken from the animals for morphological studies. The tissues were fixed with Bouin fixative for light microscopic studies and subsequently a routine paraffin embedding method was used. Liver sections of 5 μ m thickness were stained by HE and Masson's triple dyes (Masson), and examined under Carl Zeiss Ultraphot II microscope. Liver samples were fixed in 20 g/L glutaraldehyde and post-fixed in 10 g/L of phosphate-buffered osmium tetroxide. The samples were dehydrated in ethanol and embedded in epon. Specimens were examined with JEM 1011.

Immunohistochemical study

Sections were dewaxed and rehydrated. The tissues were rendered permeable with 3 g/L Triton-X 100 for 10 min and then rinsed in phosphate-buffer saline (10 mmol/L, pH 7.5). For antigen retrieval, the slides were kept in 0.01Mol/L citrate buffer for 10 min in a microwave oven. Endogenous peroxidase was blocked with 30 mL/L hydrogen peroxide. An Ultra Vision Detection System for streptavidin-biotin-peroxidase technique (Lab Vision, USA) was employed. Sections were covered with blocking serum for 10 min to block non-specific binding sites. They were then incubated with metallothionein antibody for 1 h (Zymed Laboratories, USA) at room temperature with 1:50 dilution. Slides were then incubated with PCNA mouse monoclonal antibody for 30 min (Neomarkers,

USA) at room temperature with 1:50 dilution. They were incubated for 15 min with biotinylated secondary antibody and then incubated with the streptavidin-peroxidase conjugate for 15 min. The enzyme activity was developed using aminoethylcarbazole (AEC) and then the sections were counterstained with hematoxylin. Negative control sections were prepared by substituting the metallothionein or PCNA antibodies with phosphate-buffer saline. Hepatocytes were viewed using a light microscope (Olympus, CX41) at a magnification of 400X. Approximately 400 cells from 10 randomly selected fields (0.0506 mm² per field) of vision were counted. The PCNA and metallothionein labeling indices were expressed as a percentage of positive stained cells relative to the number of counted cells. (i.e., PCNA or metallothionein labeling index = PCNA or metallothionein positive hepatocytes/total hepatocytes per high power field \times 100).

Biochemical study

In this study, biochemical investigation was carried out in serum, blood, and liver tissues. The blood samples were taken by a syringe from the heart. Blood glutathione (GSH) levels were measured according to the method of Beutler, Duron and Kelly using Ellman's reagent^[23]. Serum alkaline phosphatase activities (ALP) were determined by the Two Point method^[24]. Serum aspartate transaminase (AST) was assessed by the Reitman-Frankel method^[25]. The liver tissues were homogenized in cold 9 g/L saline by means of a glass homogenizer to make up a 100 g/L homogenate. The homogenates were centrifuged and the clear supernatants were used for GSH, lipid peroxidation (LPO) and protein levels. Liver GSH levels were determined according to Beutler's method, using Ellman's reagent^[26]. Liver LPO levels were measured by Ledwozyw's method^[27], liver protein levels were assessed by Lowry's method using bovine serum albumin as the standard^[28].

Statistical analysis

The biochemical results were evaluated using an unpaired *t*-test and analysis of variance (ANOVA) using the NCSS statistical computer package (Kaysville, Utah, USA)^[29]. The microscopic results were analyzed by one-way ANOVA followed by the Scheffé and Student's *t*-test for multiple comparisons of the control against all other groups using SPSS 13 for Windows. Data were expressed as mean \pm SD. $P < 0.05$ was considered to be significant.

RESULTS

Morphological results

The livers of control and zinc-treated rats were visually normal. Acute ethanol exposure caused degenerative morphological changes in the liver. The hepatocyte of rats exposed to absolute ethanol alone had occasional diffuse vacuolar degeneration. Vacuolar degeneration was found in hepatocytes of zones 2 and 3. In the alcohol group, there was a mild dilation of the sinusoids and hyperemia. Moreover, mononuclear cell infiltrations were evident in this group. These alcohol-induced hepatic pathological changes were significantly inhibited in the zinc-pretreated

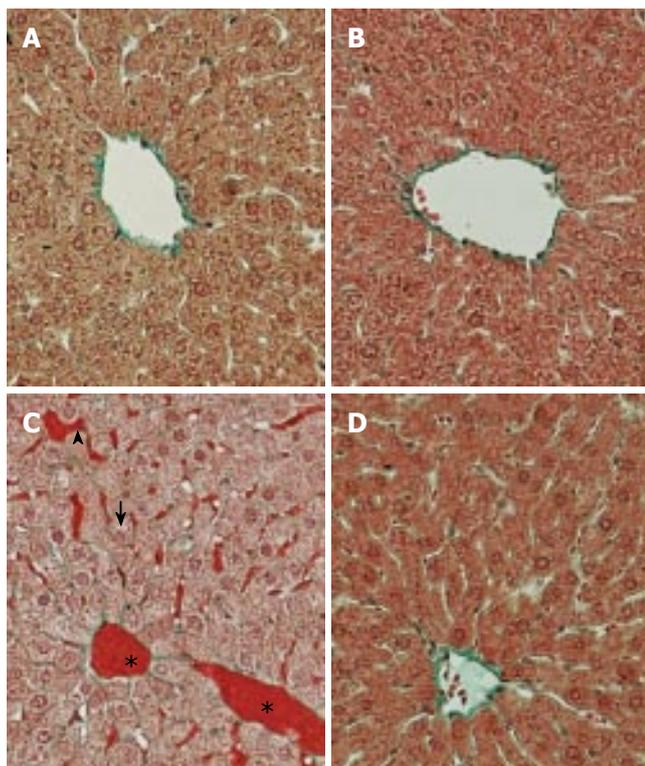


Figure 1 Histological appearance of rat liver tissue. Masson X 270. **A:** Intact control; **B:** Control, zinc sulfate; The liver showing vacuolated hepatocytes (\rightarrow) in the centrilobular area with mild dilation of sinusoids (\blacktriangleright) and hyperemia (*); **C:** Absolute ethanol; **D:** Zinc pre-treatment.

rats. A moderate degree of mucosal hyperemia was observed in the group given ethanol and zinc sulfate. In addition, less vacuolar degeneration was observed in this group (Figure 1).

An increase in hepatic metallothionein-producing cells in control rats given only zinc, when compared to intact controls, was observed. In addition, an increase in hepatic metallothionein-producing cells was observed in the rats given zinc and ethanol, when compared to the group given ethanol. Immunoreactive metallothionein-producing cells in the control group were rarely found to be scattered in lobular hepatocytes or around the periportal area. In the control rats given only zinc, more metallothionein-producing cells were observed in hepatocytes of zones 1 around the periportal area as compared with the control group. Metallothionein was observed significantly in the cytoplasm of cells by immunohistochemical staining in the rat livers of all groups. The intensity of immunoreactivity of metallothionein-producing cells in the group given ethanol and zinc was generally higher in hepatocytes of zones 1 compared to zones 2 (Figure 2). The metallothionein labeling index was determined to be $27.27\% \pm 2.02\%$ in the group given zinc sulfate, while in intact control group the index was $2.44\% \pm 0.50\%$. The metallothionein labeling index was increased 55-fold in the group administered ethanol + zinc sulfate ($26.32\% \pm 3.09\%$) compared to the group administered only ethanol ($0.48\% \pm 0.19\%$) ($^bP_{\text{test}} = 0.018$). (Figure 3A)

Hepatocyte proliferation was measured by immunohistochemical identification of PCNA. The PCNA labeling

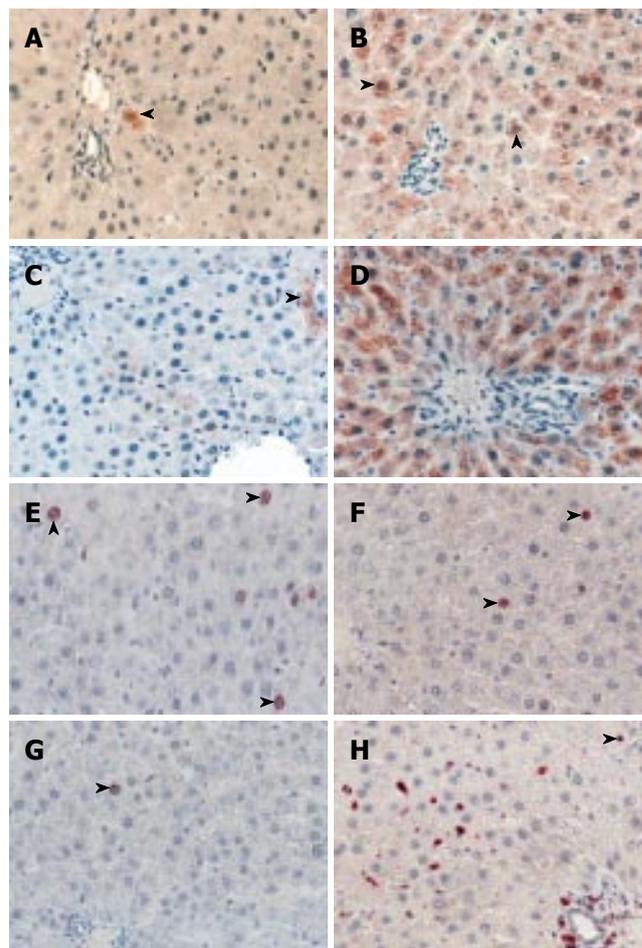


Figure 2 Immunoreactive metallothionein-producing cells (\blacktriangleright) in rat liver. Streptavidin-Biotin-Peroxidase x 270. **A:** Intact control; **B:** Control, zinc sulfate; The reduced metallothionein expression (\blacktriangleright); **C:** Ethanol; Note the increase of immunoreactivity of metallothionein-producing cells; **D:** Ethanol + zinc sulfate; **E:** Intact control, PCNA-positive hepatocytes (\blacktriangleright); **F:** Control, zinc sulfate; **G:** Ethanol; The increase in immunoreactivity of PCNA-positive cells; **H:** Ethanol + zinc sulfate.

index was determined to be $0.73\% \pm 0.17\%$ in the intact control group and $0.32\% \pm 0.02\%$ in the group given zinc sulfate. PCNA immunohistochemistry showed that the number of PCNA-positive hepatocytes was increased significantly in the livers of rats administered ethanol + zinc sulfate ($2.40\% \pm 0.57\%$) compared to the group given only ethanol ($0.19\% \pm 0.10\%$) ($^bP_{\text{test}} = 0.007$) (Figure 3B). However, according to semiquantitative evaluations, most of the PCNA-positive hepatocytes were in S phase of proliferation in all groups.

Ultrastructurally, proliferation of smooth endoplasmic reticulum, degenerative mitochondria, condensation of chromatin and increased lipid droplets were observed after alcohol treatment. However, administration of zinc sulfate caused a remarkable decrease in the lipid droplets, smooth endoplasmic reticulum, and mitochondria degeneration of the hepatocytes (Figure 4).

Biochemical results

Blood GSH levels are presented in Table 1. From the obtained results, values of GSH in the blood of the group administered ethanol showed a significant decrease

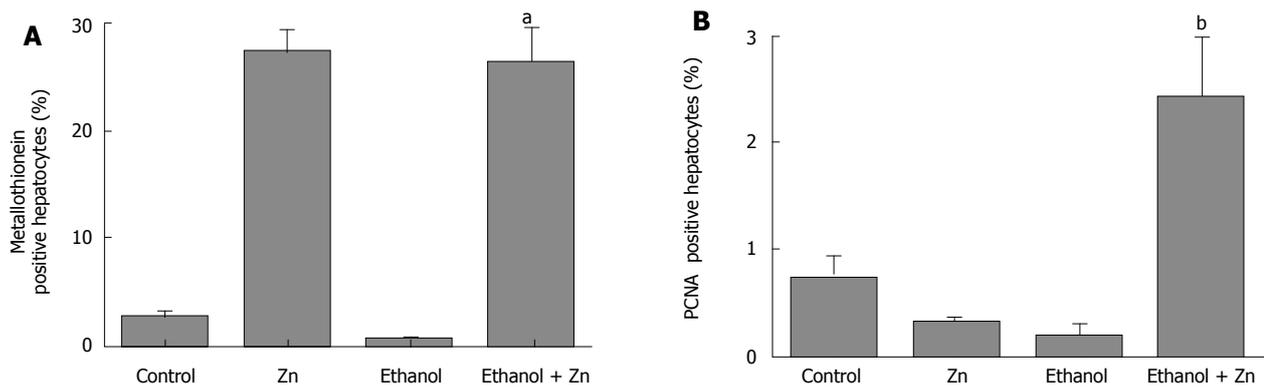


Figure 3 A: Metallothionein, ^a*P* = 0.018 vs Ethanol; B: PCNA labeling indexes, ^b*P* = 0.007 vs Ethanol.

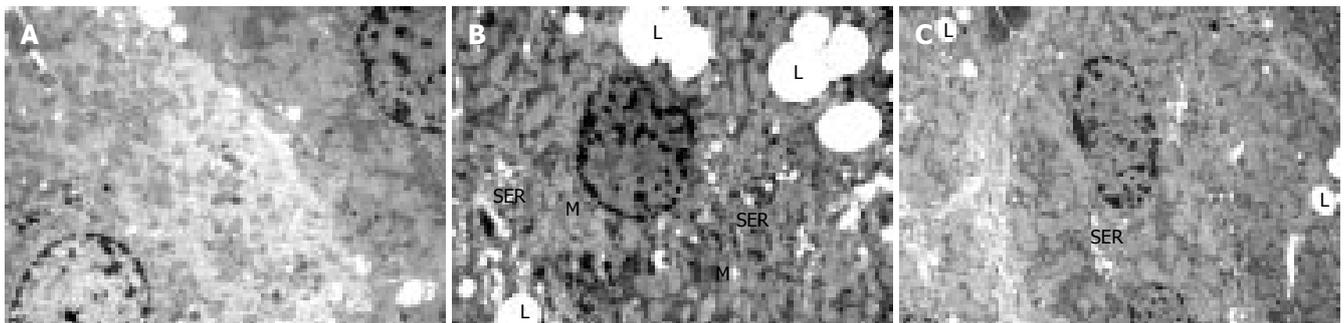


Figure 4 Ultrastructure of the hepatocytes of the rat. TEM X5000. A: Control; B: Ethanol; C: Zinc sulfate + ethanol. An increase in smooth endoplasmic reticulum (SER), lipid vacuoles (L) and degeneration in mitochondria (M) of the hepatocyte of a rat given ethanol. These ultrastructural changes were improved by zinc pre-treatment.

Table 1 Blood glutathione and serum enzymes (mean ± SD)

Group	<i>n</i>	GSH (mg %)	<i>P</i> -test	ALP (U/L)	<i>P</i> -test	AST(U/L)	<i>P</i> -test
Control	8	52.59 ± 2.44		101.12 ± 1.64		122.55 ± 2.61	
Control + Zinc sulfate	8	46.65 ± 4.90		73.91 ± 6.28	0.0001	106.03 ± 8.97	0.001
Ethanol	14	35.09 ± 2.31 ^b	0.005	110.02 ± 2.88 ^b	0.0001	133.00 ± 4.82 ^b	0.0001
Ethanol + Zinc sulfate	11	38.39 ± 6.18		84.80 ± 7.09		112.56 ± 8.18	
<i>P</i> _{ANOVA}		0.0001		0.0001		0.0001	

^b*P*-test = 0.0001 vs Control.

when compared to the control group (^b*P*-test = 0.0001). Also, a marked increase in blood GSH level was observed in the group administered ethanol + zinc sulfate when compared to the group administered ethanol (*P*-test = 0.005). According to Table 1, a significant difference in the blood GSH levels of four groups was observed (*P*_{ANOVA} = 0.0001).

Serum ALP and AST activities are given in Table 1. From the obtained results, values of ALP in the serum of the group administered ethanol showed a notable increase when compared to the control group (^b*P*-test = 0.0001). Also a considerable decrease was noted in the group administered ethanol + zinc sulfate when compared to the group administered ethanol (*P*-test = 0.0001). According to Table 1, a significant difference in the ALP activities of the four groups were observed (*P*_{ANOVA} = 0.0001). In this study,

a statistically marked increase was observed in serum AST activity of the group administered ethanol, in comparison with the control group (^b*P*-test = 0.0001). On the other hand, in the group to which ethanol + zinc sulfate was administered, the AST activity decreased compared to the ethanol group. A significant difference in the serum AST activities of four groups was observed (*P*_{ANOVA} = 0.0001).

Table 2 shows the effects of zinc sulfate on liver GSH and LPO. The GSH levels were significantly reduced in the group administered ethanol as compared to the other groups (*P*_{ANOVA} = 0.0001). A significant decrease of liver GSH levels in the group administered ethanol was determined in comparison to the control group (^b*P*-test = 0.0001). After zinc sulfate administration to ethanol-treated rats, liver GSH levels increased greatly when compared

Table 2 Effects of zinc sulfate on the levels of lipid peroxidation and glutathione in the liver (Mean \pm SD)

Group	n	GSH nmol GSH/mg protein	$P_{t\text{-test}}$	LPO nmol MDA/mg protein	$P_{t\text{-test}}$
Control	8	16.64 \pm 1.24	0.589	0.52 \pm 0.12	0.065
Control + Zinc sulfate	8	15.84 \pm 3.87		0.69 \pm 0.06	
Ethanol	14	12.23 \pm 2.92 ^b	0.0001	0.93 \pm 0.06 ^b	0.001
Ethanol + Zinc sulfate	11	20.74 \pm 5.11		0.51 \pm 0.10	
P_{ANOVA}		0.0001		0.0001	

^b $P_{t\text{-test}} = 0.0001$ vs Control.

to the ethanol group ($P_{t\text{-test}} = 0.0001$). Zinc sulfate had no significant effect on liver GSH levels in the control group ($P_{t\text{-test}} = 0.589$). An eminent difference in the liver LPO levels of the four groups was observed ($P_{ANOVA} = 0.0001$). A significant increase of liver LPO levels in the group administered ethanol was determined in comparison to the control group ($P_{t\text{-test}} = 0.0001$). After the administration of ethanol + zinc sulfate, the liver LPO levels decreased notably when compared to the ethanol group ($P_{t\text{-test}} = 0.001$).

DISCUSSION

Ethanol-induced diseases in humans are important in clinical gastroenterology. Animal models in alcohol research have already been applied to study acute and chronic ethanol damage of the liver. Ethanol may accelerate oxidative stress *via* increased production of active oxygen species. Ethanol-induced oxidative stress plays a major role in liver injury^[30]. Ethanol causes a notable fall in the level of zinc in the liver^[1]. Zinc may play a key role in certain alterations observed in alcoholic patients^[31]. The antioxidant defensive system of the liver might be influenced by changes in the zinc content of the liver^[16]. Floersheim^[32] suggested that zinc salts reduce tissue injury caused by a free radical-mediated mechanism. The present study suggests that ethanol-induced hepatic injury is related to the formation of free radicals and pretreatment of zinc prior to the administration of ethanol prevents toxicity. Ethanol treatment is accompanied by the generation of free radicals which stimulate LPO and decrease GSH levels. Therefore, zinc supplementation prevented ethanol-induced decreases in GSH and increases in LPO.

Under light and electron microscopes, the decrease of degenerative changes in the livers of rats administered ethanol + zinc sulfate indicates that zinc ameliorates the damage in the liver tissue of the group given ethanol. An increase in hepatic metallothionein-producing cells in control rats given only zinc as compared to intact controls was observed. This increase shows that metallothionein is required for high zinc levels in liver. In this study, synthesis of metallothionein was shown to increase in the livers of rats given zinc + ethanol, immunohistochemically, in accordance with previous findings^[33]. Our results

indicate that metallothionein induction by zinc sulfate has a protective effect against the injury of acute ethanol administration in liver. Elevation of metallothionein may maintain the integrity of the membrane of the organelles, as observed in the group given ethanol + zinc sulfate. Metallothionein expression may vary with types of tissue and physiological and nutritional factors such as zinc. There are limited studies on hepatic metallothionein levels following acute administration of ethanol to rats^[4,11]. An over-expression of metallothionein serves to protect cells from the alkylating agents^[14]. In the ethanol-intoxicated group, metallothionein levels increased nearly 3.5-times when compared to the control group^[7]. Ebadi *et al*^[22] also reported that the administration of zinc sulfate increased the synthesis of metallothionein mRNAs. It has been reported that hepatic metallothionein synthesis is stimulated by dietary zinc supplementation^[34]. Koterov^[11] reported that there was a linear dependence of the hepatic levels of metallothioneins with the dosage of alcohol. However, recent studies have shown that zinc inhibition of acute alcohol-induced liver injury is independent of metallothionein^[35,36]. In the present report, the metallothionein labeling index correlated positively with the PCNA index in the rats given zinc + ethanol. It is suggested that enhanced antioxidant proteins such as metallothionein may be involved in hepatocyte proliferation. This finding supports a previous study showing an impairment of liver regeneration in metallothionein-I and metallothionein-II gene knockout mice after partial hepatectomy^[37].

The liver is the most important organ in alcohol metabolism. The increase in AST and ALP activities in serum is an indicator of liver destruction. Their increase in the serum activities of these enzymes was directly proportional to the degree of cellular damage. The activity of AST in the serum of the group administered ethanol showed a statistically significant increase when compared to the control group. AST activity decreased in the group administered ethanol + zinc sulfate. This suggests that zinc pretreatment of rats prevented the elevation of serum AST. Acute ethanol exposure caused a 4-fold increase in the levels of serum AST compared with control animals^[3]. The activity of ALP in the serum of the group administered ethanol has shown a significant increase when compared to the control group. The increase of alkaline phosphatase in the serum may be a result of damage to liver cells. Since zinc is a component of many enzymes, including alkaline phosphatase, a significant decrease was found in the activity of ALP in the group administered ethanol + zinc sulfate when compared to the group administered ethanol.

Glutathione plays a major role as a reductant in oxidation-reduction processes and also serves in detoxification^[38]. Ethanol, or its metabolites, can alter the balance in the liver toward auto-oxidation, either acting as pro-oxidants, or reducing the antioxidant levels, or both^[39]. The pathogenesis of alcohol-induced liver disease involves the adverse effect of ethanol metabolites and oxidative tissue injury^[40]. The most prominent defensive system in the liver is reduced GSH. The values of GSH in blood of the rats administered ethanol have shown a significant decrease when compared to the control

group. Metallothionein shares an important similarity with glutathione due to the fact that one-third of their amino acids are cysteines. Experimental depletion of glutathione in isolated rat hepatocytes has been shown to induce metallothionein expression and to create a new pool of thiol groups in the cell^[41]. Glutathione provides a protective action against damage from reactive oxygen species and free radicals formed during drug metabolism^[42,43]. On the other hand, Dreosti *et al*^[44] reported that ethanol alone had no effect on glutathione levels. Cho *et al*^[45] reported that ethanol and zinc sulfate administration did not affect hepatic glutathione levels in mice. It is reported that zinc supplementation prevented ethanol-induced decreases in glutathione concentration in the liver^[36], in accordance with our findings.

Chronic ethanol administration induces oxidative stress and increases lipid peroxidation of the cell membrane. This leads to increased membrane fluidity, disturbances of calcium homeostasis, and finally results in cell death^[46]. Oxidative stress is characterized by increased lipid peroxidation. A remarkable increase of liver lipid peroxidation levels in the group administered ethanol was determined in comparison to the control group. The present results suggest that zinc sulfate supplementation has a protective effect against lipid peroxidation in liver. Lipid peroxidation was observed to increase prominently in ethanol-fed rats after 4 and 8 wk when compared to the controls^[47]. Patients and experimental animals with acute and chronic ethanol exposure have a depleted liver glutathione content, correlated with an increase in lipid peroxidation^[48,49]. Zinc supplementation for 12 wk caused a decrease in lipid peroxidation, together with an increase in metallothionein concentration in rats^[50]. However, 100 mL/L ethanol ingestion for 8 wk enhanced lipid peroxidation in liver^[51]. Cabre *et al*^[50] reported that zinc is an efficient hepato-protective agent against lipid peroxidation in alcoholic rats.

In conclusion, microscopic and biochemical evaluations reveal that zinc sulfate has a protective effect on ethanol-induced liver injury. Our results demonstrate that zinc acts as an antioxidant agent in hepatic antioxidant systems after acute ethanol administration. In addition, this protective effect against acute ethanol injury has also included proliferation in hepatocytes. Zinc may be a therapeutic agent in the prevention and treatment of ethanol-induced liver injury.

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BASIC RESEARCH

Influences of enteral nutrition combined with probiotics on gut microflora and barrier function of rats with abdominal infection

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Abstract

AIM: To investigate the influences of enteral, parenteral nutrition and probiotics delivered by gut on intestinal microecology, epithelial tight junctions, immune and barrier function of rats with abdominal infection.

METHODS: Rat abdominal infection models established with cecal ligation and perforation method, were divided into three groups: parenteral nutrition (PN group, $n = 7$), PN+enteral nutrition (EN group, $n = 7$) and PN + EN + probiotics (probiotics group, $n = 7$) *via* the needle jejunostomy and neck vein for five days. The total nutritional supplement of the three groups was isonitrogenic and isocaloric. Probiotics was delivered by jejunostomy 10 mL/d (1×10^8 cfu/mL). The rats were killed on the sixth day. The feces in the cecum were cultured for anaerobic bacterial growth and analyzed with bacterial group DNA fingerprint profile with random amplified polymorphic DNA. The transmembrane binding proteins (occludin) and IgA level in plasma cells of intestine epithelium in colon and terminal ileum were measured by an immunohistochemistry method. The ultrastructure of intestinal epithelial tight junctions in colon and small intestine was observed by electron-microscopy. Vena cava blood and the homogenated tissue of liver, lung and mesenteric lymph nodes were cultured to determine the bacterial translocations, and endotoxin in the blood from portal vein was detected.

RESULTS: (1) The amount of bacteria of gut species in EN group and probiotic group was higher than that in PN group. The DNA-profiles in EN group and probiotic group were similar to that of normal rats. The number of DNA-profiles in probiotics group was much more than that in PN group and EN group. Moreover, there were strange stripes in PN group. (2) The expression of occludin and

IgA in the small and large intestine in EN group (2.309 ± 0.336 , 15.440 ± 2.383) and probiotic group (2.938 ± 0.515 , 16.230 ± 3.183) was improved as compared with PN group (1.207 ± 0.587 , $P < 0.05$, 11.189 ± 2.108 , $P < 0.01$). The expression of occludin in probiotic group (intestine: 2.93 ± 0.515 ; cecum: 3.40 ± 0.617) was higher than that in EN group (intestine: 2.309 ± 0.336 ; cecum: 2.076 ± 0.670 ; $P < 0.05$). The expression of IgA, especially in EN group (intestine: 15.440 ± 2.383) and probiotic EN group (large intestine: 12.516 ± 1.542) significantly increased as compared with PN group (intestine: 11.189 ± 2.108 ; cecum: 10.160 ± 1.643 ; $P < 0.01$). The intestinal epithelial tight junctions and microvilli of the probiotic group were more intact than those in the PN group. (3) The bacterial translocations in blood, liver, lung and mesenteric lymph nodes, and the levels of endotoxin were significantly reduced in probiotic (0.082 ± 0.029) and EN (0.125 ± 0.040) groups as compared with PN group (0.403 ± 0.181 , $P < 0.05$).

CONCLUSION: Application of EN combined with probiotics could improve the expression of transmembrane binding proteins (occludin) and IgA, correct the intestinal flora disturbance, maintain gut barrier functions and tight junctions, and reduce the occurrence of gut bacterial translocation.

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Key words: Probiotics; Enteral nutrition; Gut flora; Transmembrane binding proteins; Gut barrier function

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INTRODUCTION

Since Deitch^[1] put forward the concept of bacterial translocation in the 1980s, it has been commonly accepted that bacteria and endotoxin translocations are always associated with the destruction of gut barrier, which could also cause systemic infection reactive syndrome (SIRS), pyemia, shock, and multi-organs dysfunction syndrome (MODS), and increase the death rate of patients

with severe illness. Hence, the mechanism of bacterial translocation and the protective and therapeutic agents, as well as the relation between gut original infection and bacteria translocation and their influence on intestinal function have attracted much clinical attention. Though development of new antibiotics has made much progress, the death rate in MODS is still quite high. Under such circumstances, some scholars proposed that the therapy of infection should be directed at its "source" not its "target"^[2]. And methods such as taking the unabsorbed antibiotics orally, implementing enteral nutrition at early stage, and decontaminating the intestine were used to help recover the physiology state of intestinal tract, to reduce the possibility of gut microflora disorder and bacterial translocation and maintain normal gut function.

In recent years, researchers have tried to improve the gut microbial and bacterial translocation by probiotics^[3,4]. However, the thorough and detailed mechanisms are lacking. The present study aimed to determine the effect of probiotics on the ultrastructure of tight junctions, transmembrane protein-occludin expression, enterocyte IgA levels, gut mucus form, gut microflora and bacterial translocation by parenteral nutrition (PN), enteral nutrition (EN) and enteral nutrition combined with probiotics supplied to rats with abdominal infection. It will provide experimental evidence for using probiotics in clinical therapy of abdominal infections.

MATERIALS AND METHODS

The abdominal infection-PN and EN rats model

Fifty-eight SD rats weighing 250-320 g (Fudan University Medical Animal Center, Shanghai) were anesthetized with intraperitoneal injections of 2% saline-ketamin injection liquid (30 mg/g weight). The rats were fixed in a supine position and prepared in a sterile manner for catheterization and operation. A silastic catheter (0.6 mm in inner diameter, 1.0 mm in outer diameter) was inserted through the external jugular vein into the superior vena cava. The catheter was tunneled subcutaneously to the midscapular region and guarded by the flexible spring, and then hooked up to an infusion pump (B. Braun). The abdominal infection model was established by cecum perforation and ligation (CPL)^[5], here one third of the cecum distal was ligated and perforated with holes through which a piece of rubber patch was placed and the pinhead hole (#12) fixed. A silastic catheter (1.2 mm in diameter) was placed by jejunostomy 30-40 cm away from the cecum, and the catheter was tunneled abdominally subcutaneously to the back. The rats were maintained in individual metabolic cages^[6]. The silastic catheters of jejunum and neck vein were traversed together through the spring and linked to the infusion pump on the circumrotate equipment^[7]. The PN and EN nutrient solution was infused at a constant infusion rate by 2 pumps for 5 d during the experimental period.

Animal grouping

After establishment of model, all rats received intravenous 0.9% saline solution at 2.0 mL/h for 24 h. Rats were divided into three groups, PN group ($n = 7$): supported

Table 1 PN solution prepared (contents/100 mL)

Prescription	Dose
50% Glucose (mL)	33
8.5% Novamin (mL)	45
20% Intralipid (mL)	17
Solvita (mL)	1
Addamel (mL)	1
10% KCl (mL)	1
10% NaCl (mL)	1
RI (U)	4
Heparin (U)	40
Non-protein calorie (kJ)	348
Total nitrogen (mg)	504

by PN; PN + EN group (EN group, $n = 7$): supported by PN + EN(peptide), EN was dripped through jejunum tube; PN + EN + probiotic group (probiotic group, $n = 7$): supported by PN + EN(peptide), the probiotics was infused by jejunostomy tube at about 10 mL/d. The 3 groups were isonitrogenic and isocaloric (Table 1) during 1-5 d. In the PN group, 100% calorie and nitrogen were supplied by the PN solution at the infusion speed of 3.3 mL/h and 80 mL/d. In EN groups, 80% calorie and nitrogen were supplied by the PN solution at an infusion speed of 2.6 mL/h, and 64 mL/d, and the other 20% were supported by EN (Pepti-2000) at 16 mL/d, which were diluted 2:1 to 24 mL with saline solution and the infusion speed maintained at 1 mL/h. In the probiotic group, the probiotics (supported by Shanghai Jiaotong University Limited Company) were added at 10 mL/d. The daily dose of amino acids was 2.5 g of nitrogen per kilogram, the amount of non-protein calories was 348 kJ/d and the total nitrogen was 504 mg/d. The rats were killed and samples taken in the morning of the 6th d.

Probiotics containing live lactobacillus plantarum (activity 1×10^8 cfu/mL) was infused at about 10 mL/d by jejunostomy tube twice per day through 1-5 d.

Fecal bacterial anaerobic culture

The fecal sample (0.1 g) in cecum was placed in 9 mL Ringer dilution solution(which contained cysteine) and incubated anaerobically at $36 \pm 1^\circ\text{C}$ supplemented with brucella blood agar (2 d, total anaerobic CFU), bacteroides bile esculin agar (2 d), egg yolk agar (after equal volumes of 95% ethanol were added to the dilutions and the preparations stood for 30 min to select for clostridial spores), and Rogosa SL agar (Difco) (2 d for lactobacilli and 4 d for bifidobacteria, after Lactobacillus colonies were marked at 2 d). The dilutions were removed from the anaerobic glove box and inoculated (100 μL inocula) into plates which contained the following media: supplementary brucella blood agar (2 d, total aerobic CFU), MacConkey agar (Difco) (1 d, enterobacteria), bile esculin azide agar (Difco) (1 d, Cl Perfringens) and were incubated aerobically at 37°C . To analyze the total Lactobacillus population, 10 colonies were picked randomly from a dilution agar plate containing about 100 colonies. The bacterial colonies were counted and identified by Microscan Autoscan-4 Machine (Dade Behringcom).

Gut bacterial DNA fingerprint profiles

The fecal samples in cecum from each subject were also examined by PCR-denaturing gradient gel electrophoresis (DGGE) profiles. To extract bacterial DNA, 1 mL of fecal homogenate in pH 7.0 phosphate buffer (the buffer used for the azoreductase assay) was centrifuged at 14 600 g for 5 min (5°C). DNA was extracted from the resulting pellet with a Fast DNA kit (BIO 101, Vista, CA) by using CLS-TC (a cell lysis solution used for animal tissues and bacteria). The V2-V3 region of the 16S rDNA gene (positions from 339 to 539 in the *Escherichia coli* gene) of bacteria in the fecal samples was amplified by using primers bacteria ITS PS2 (5'-TG(C/T) ACA CACCGC CCG T-3'), PL2 (5'-GGG T (G/C/T) CCC CAT TC(A/G)G-3'). PCR was performed with 0.2-mL tubes by using a PCR Express thermal cycler (Hybaid, Teddington, UK). Each reaction mixture (50 µL) contained reaction buffer (10 mmol/L [final concentration] Tris-HCl, 2.5 mmol/L [final concentration] MgCl₂, 50 mmol/L [final concentration] KCl [pH 8.3]), each deoxynucleoside triphosphate at a concentration of 200 µmol/L, 20 pmol of each primer, 1 µL of fecal DNA, and 2.5 U of Taq DNA polymerase (Boehringer, Mannheim, Germany). The following amplification program was used: 94°C for 3 min, followed by 30 cycles consisting of 94°C for 30 s, 56°C for 30 s, and 68°C for 60 s, and finally 7 min at 68°C. DGGE was performed by using a DCode universal mutation detection system (Bio-Rad, Richmond, CA) and gels that were 16 cm × 16 cm × 1 mm; 6% polyacrylamide gels were prepared and electrophoresed with 1 × TAE buffer prepared from 50 × TAE buffer (2 mol/L Tris base, 1 mol/L glacial acetic acid, 50 mmol/L EDTA). The denaturing gradient was formed by using two 6% acrylamide (acrylamide/bisacrylamide ratio, 37.5:1) stock solutions (Bio-Rad). The gels contained a 22%-55% gradient of urea and formamide that increased in the direction of electrophoresis. A 100% denaturing solution contained 400 mL/L formamide and 7.0 mol/L urea. Electrophoresis was performed at 130 V (constant voltage) and 60°C for about 4.5 h. Electrophoresis was stopped when a xylene cyanol dye marker reached the bottom of a gel. The gels were stained with an ethidium bromide solution (5 µg/mL) for 20 min, washed with deionized water, and reviewed by UV transillumination^[8,9].

Gut mucosa occludin and IgA indirect-immune fluorescence expression

The distal ileum and colon tissue were taken to determine the occludin and IgA expression by indirect-immune histochemical fluorescence.

Occludin: The tissue was embedded into the frozen acetone at -20°C. Then it was sliced, and routinely dehydrated. To remove the endogenous peroxidase enzyme activity, the slides were treated with 0.1%-1% H₂O₂ at 37°C for 5-10 min. Then they were washed with PBS twice, and incubated at room temperature overnight with primary antibody 1:300 (goat anti-rat occludin antibody, Santa Cruz). And then they were washed with PBS 3 times and incubated with the second antibody 1:200 (anti-goat IgG, Santa Cruz) for 30 min, then washed again with PBS 3 times and the formula AB enzyme reagent was added.

Then they were washed with PBS again and incubated with peroxidase enzyme for 30 s-10 min. After that, the slides were washed by H₂O₂, dehydrated and mounted.

IgA: The tissue was embedded into paraffin and sliced, then routinely dewaxed. The slides were washed with PBS 3 times, incubated with 3.3% H₂O₂ at room temperature for 10-15 min to block the endogenous peroxidase enzyme, then washed with PBS again, and albumin interdiction solution was added for 5 min to reduce the nonspecific background staining. Then slides were washed with PBS, and the first antibody 1:500 (mouse anti-rat IgA antibody, Sigma) was added at 4°C refrigerator overnight. After that slides were washed with PBS again, and second antibody 1:100 (anti-mouse antibody, DAKO) labeled with biotin was added. Slides were then washed with PBS again, and visualised with DBA enzyme-substrate illustration system, then dyed again and developed.

The data were analyzed by HPIAS1000 high definition color image manipulation system. Under the same expanding multiple (400), the occludin density was determined per field of vision by light-densimeter (at least five fields per slide).

Tight junction of ileum and cecum by electron microscopy

The gut tissues about 0.5 cm in length were taken from distal ileum and colon 5 cm away from the valva ileocaecalis. One fixation procedure was used for conventional thin-section electron microscopy. The fixation procedure included incubation with OsO₄ alone (1% or 2% in phosphate buffer) at 0°C for 30 min. After fixation, the ileum and cecum were washed extensively in Veronal acetate buffer (90 mmol/L, pH 6.0), stained by incubation at 0°C for 60 min in uranyl-magnesium acetate (0.5%) in the same buffer, washed again, dehydrated, and embedded. Thin sections were doubly stained with uranyl acetate and lead nitrate and the change of tight junction and microvilli were observed (2 × 10⁴) using a Philip EM 400 electron microscope.

Bacterial translocation rate of mesenteric lymph node and remote organ

Blood from vena cava (1 mL), mesenteric lymphatic tissue (5 g), liver and lung tissue, were collected, homogenized and placed into a tube containing cardio-cerebral leachate, incubated at 35°C. After 18-24 h samples were stained with methylene blue-eosin method, and continued to be cultured at 35°C. 18-24 h later, the number of bacterium was counted by Microscan Autoscan-4 machine.

Endotoxin

Blood from the portal vein (1-2 mL) was taken into heparin lithium anticoagulant centrifuged at 500 r/min for 5 min. The supernatants were mixed with the limuluslysate reagent to examine the endotoxin level.

Statistical analysis

Analysis of data was performed by χ^2 and Dunnette *t* test with statistical program SPSS10.0, the experimental data were expressed as mean ± SD of the samples between 3 groups. The level of significance was set at $P < 0.05$ or $P < 0.01$.

Table 2 Enteral nutrition (Pepti-2000 variant¹) composition (126 g/500mL)

Ingredients	Contents
Protein(g)	19.9
Protein hydrolysate (g)	19.9
Nitrogen (g)	2.9
Fat (g)	4.9
Vegetable oil (g)	2.45
MCT (g)	2.45
Linoleic acid	1.3
Carbohydrates (g)	93.1
Glucose (g)	1.8
Maltose (g)	5.5
Amylose (g)	84.4
Lactose (g)	< 1.3
Organic acid (g)	0.1
Minerals (g)	2.6
Vitamins (g)	0.4
Nonprotein energy (kJ)	2090
Protein (En%)	16
Fat (En%)	9
Carbohydrates (En%)	75
Osmotic pressure (mOsm/L)	410
PH	6.0

¹A commercial, nutritional complete, short-chain peptide based elemental diet (Nutricia, The Netherlands).

RESULTS

General condition and death rate

The CPL model was established successfully in 58 SD rats; 37 rats died, with the total death rate during 6 d of 63.8%. The death rate in PN group (78.7%) was higher than that in probiotic group (41.6%, $\chi^2 = 5.658$, $P = 0.0174 < 0.05$, Table 2) and EN group (46.1%, $\chi^2 = 4.691$, $P = 0.030 < 0.05$, Table 3). Interloop abscess (70%), liver abscess (35%) and lung abscess (20%) were observed in the surviving rats, and the positive rate of the blood culture reached 37%.

Amount of fecal bacteria in the cecum

In the cecum, the amount of all genus (Enterobacillus, $t = -0.276$, $P = 0.018 < 0.05$, Bifidobacteria, $t = -2.749$, $P = 0.017 < 0.05$, Lactobacillus, $t = -8.033$, $P = 0.002 < 0.01$, Cl Perfringens, $t = -2.317$, $P = 0.031 < 0.05$) of the EN group were more than that of the PN group. In the probiotic group the amount of Enterobacillus ($t = -2.30$, $P = 0.390 < 0.05$), lactobacillus ($t = -9.935$, $P = 0.001 < 0.01$) and bifidobacteria ($t = -5.644$, $P = 0.002 < 0.01$) increased, and the amount of Cl Perfringens ($t = -3.144$, $P = 0.384 < 0.05$) decreased as compared with PN group.

DNA fingerprint profiles

The profiles of DNA sequence in PN group on electrophoresis (lanes 3-9) were less than that in the normal group (lanes 1-2), EN group (lanes 10-16), and probiotic group (lanes 17-23); lanes 3, 6, 7 presented with a new 16S rDNA sequence in the profile (labeled in Figure 1). The sequence profiles of expression in probiotics and EN groups were

Table 3 Death rate in celiac infection model of three groups

Group	n	Death	Death rate
PN	33	26	78.7%
EN	13	6	46.1% ^a
Probiotic	12	5	41.6% ^a

^a $P < 0.05$ vs PN group.

similar to that in the normal group (lanes 9-10).

Gut mucosa occludin and IgA expression

There was more occludin expression in the probiotic group and EN group on the surface, intracellular and inter-cell spaces of the gut epithelial cells as compared with PN group (Figures 2A-F). The occludin positive expression area per measured-window in EN group (intestine, $t = -4.955$, $P = 0.034 < 0.05$, cecum, $t = -5.407$, $P = 0.019 < 0.05$, Table 4) and probiotics group were higher than that in PN group (intestine, $t = -5.426$, $P = 0.002 < 0.01$, cecum, $t = -6.112$, $P = 0.006 < 0.01$), and that in the probiotic group was higher than that in EN group (intestine, $t = -2.645$, $P = 0.023 < 0.05$, cecum, $t = -2.307$, $P = 0.042 < 0.05$).

The expression of IgA in EN group (intestine, $t = -4.995$, $P = 0.003 < 0.01$, cecum, $t = -2.850$, $P = 0.014 < 0.05$) and probiotic group (intestine, $t = -3.575$, $P = 0.024 < 0.05$, cecum, $t = -4.946$, $P = 0.004 < 0.01$) was higher than that in PN group, especially the small intestine of the EN group and the cecum of the probiotic group.

Tight junction and microvilli micro-structure

There were more integrated tight junctions, less mitochondrion endoplasm and microvilli brushed in EN group and probiotics group as compared with PN group (Figures 3A-F). The readability of the tight junction in probiotics group was clearer than that in PN group and EN group.

Endotoxin and bacterium translocation rate of mesentery lymph node (MLN) and remote organ

Bacterium translocation rate (BTR) in PN group (60.7%) was higher than that in probiotic group (28.6%, $\chi^2 = 5.853$, $P = 0.016 < 0.05$, Table 5) and EN group (32.1%, $\chi^2 = 4.595$, $P = 0.032 < 0.05$, Table 5), whereas there was no marked difference of the BTR between the probiotics group and EN group ($\chi^2 = 0.0845$, $P = 0.7713 > 0.05$).

The endotoxin level of the PN group was higher than that in EN group ($t = 3.954$, $P = 0.002 < 0.01$) and in probiotic group ($t = 4.615$, $P = 0.001 < 0.01$).

DISCUSSION

PN has achieved significant effect as an important therapeutic method for patients with severe illness. However, long-term utility of PN may induce injury of the gut barrier and bacteria translocation. On the other hand, many therapeutic approaches including enteral nutrition and glutamine have been used to improve gut barrier function and positive results have been reported.

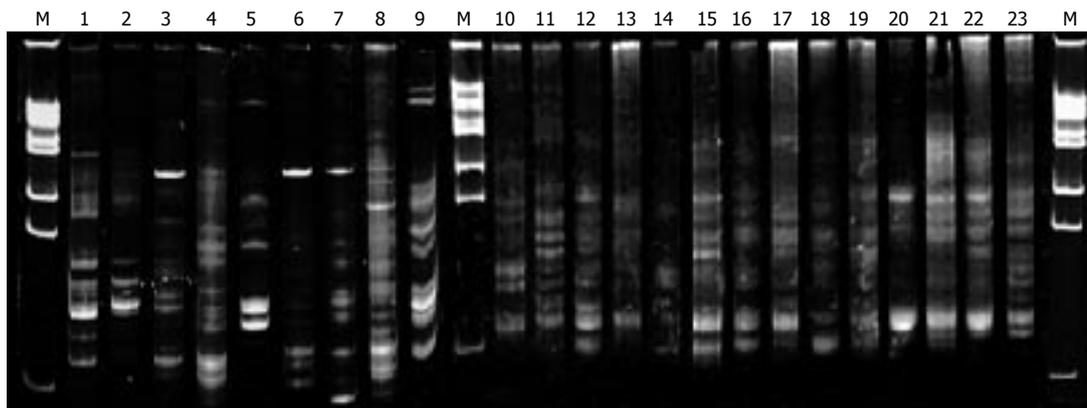


Figure 1 Gut bacterium colony DNA fingerprint profiles. Lane M: Marker; lanes 1-2: normal group; lanes 3-9: PN group; lanes 10-16: EN group; lanes 17-23: Probiotic group.

Table 4 Occludin and IgA positive expression in three groups

Group	n	Intestine (%)		Cecum (%)	
		Occludin	IgA	Occludin	IgA
PN	7	1.207 ± 0.587	11.189 ± 2.108	1.254 ± 0.203	10.160 ± 1.643
EN	7	2.309 ± 0.336 ^a	15.440 ± 2.383 ^b	2.076 ± 0.670 ^a	12.516 ± 1.542 ^a
Probiotic	7	2.93 ± 0.515 ^b	16.230 ± 3.183 ^a	3.40 ± 0.617 ^b	14.604 ± 1.837 ^b

^aP < 0.05 vs PN group; ^bP < 0.01 vs PN group.

Nevertheless, they all have certain shortfalls and defects, especially regarding their effect on the gut microbiota^[6]. With stress impairments such as wounding, surgery, and infection, not only is the gut barrier disrupted, but normal gut microbiota is also destroyed due to the change in the normal physiology of the gut and the use of antibiotics. The conditional pathogen can predominantly reproduce, disperse and translocate to extra intestinal organs and disseminate throughout the body through blood circulation. As the probiotics are autochthonous habitual bacteria, extra supplement with them could inhibit the potential pathogen's activity by adhering and colonizing to the intestinal epithelial cell, depressing the pathogenicity of the pathogens, and restoring the normal physiology of the gut. Utility of microorganism regulators such as lactobacillus, bifidobacteria, enterococci and the toxinless, harmless and safe aerophilic bacillus could improve the microflora, reduce the bacterial translocation and protect the gut barrier, and satisfactory results have been reported^[7]. Considering the specific effect of the probiotics, Bengmark^[10] proposed a new concept of econutrition in 1996, suggesting that. Adding the probiotics to the traditional enteral nutrition, can reduce the excess growth of the pathogen by its antagonist action, improve the environment of the gut by enhancing gut microflora's fermenting activity, thus maintaining the intestinal function and the gut microbiology, improving the nutritional state and immune capability against disease, reducing the infection rate of the patients with critical illness⁷.

In the current study, a model of rats with abdominal infection was established to observe the influences of different nutritional pathway and probiotics on the gut ecology and barrier function by CLP method. In traditional fecal anaerobic cultivation, gut microflora

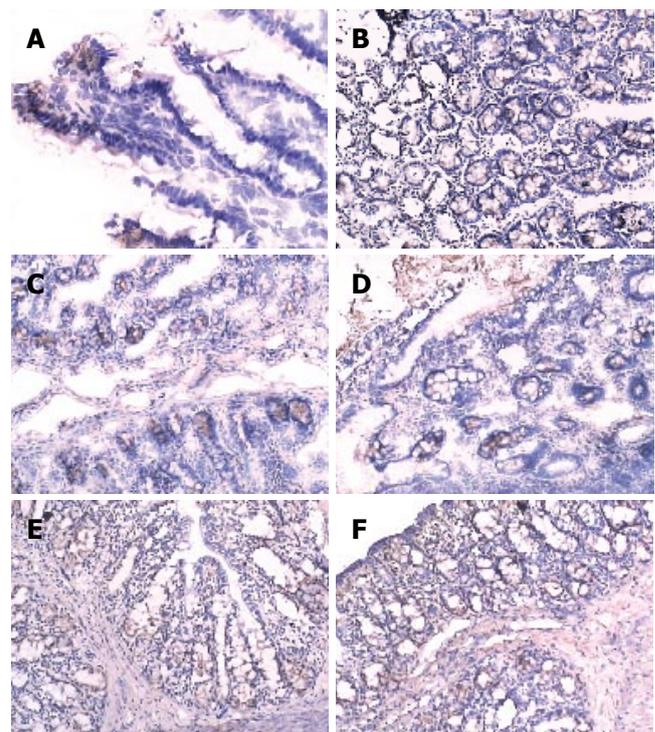


Figure 2 Intestinal and colon epithelial occludin expression. **A:** Intestinal epithelium occludin expression in PN group (200 ×); **B:** decreased epithelium occludin expression in colon in PN group (200 ×); **C:** Intestinal epithelium occludin expression in probiotics group (200 ×); **D:** Colon epithelium occludin expression in probiotics group (200 ×); **E:** Intestinal epithelium occludin expression in probiotics group (200 ×); **F:** Colon epithelium occludin expression in probiotics group (200 ×).

disorder was observed and conditional pathogenic bacteria (Cl Perfringens) increased markedly in PN group. Microflora partly recovered in EN group because of the effect of the EN itself. Both the amount and the species were more than that of the PN group, but the number of conditional pathogen was increased also. However, while the amount of lactobacillus and bifidobacteria increased by adding the probiotic, the Cl Perfringens were inhibited effectively. Meanwhile the bacteria of normal rats were logarithm mean of the major bacteria: Enterobacillus (5.732), lactobacillus (6.903), bifidobacteria (8.941), Cl Perfringens (5.132). The amount of gut flora in the probiotic group was closer to that of normal rats, suggesting the relevant intestinal pathogens could be controlled using probiotics. In this study, it was difficult to identify the type or

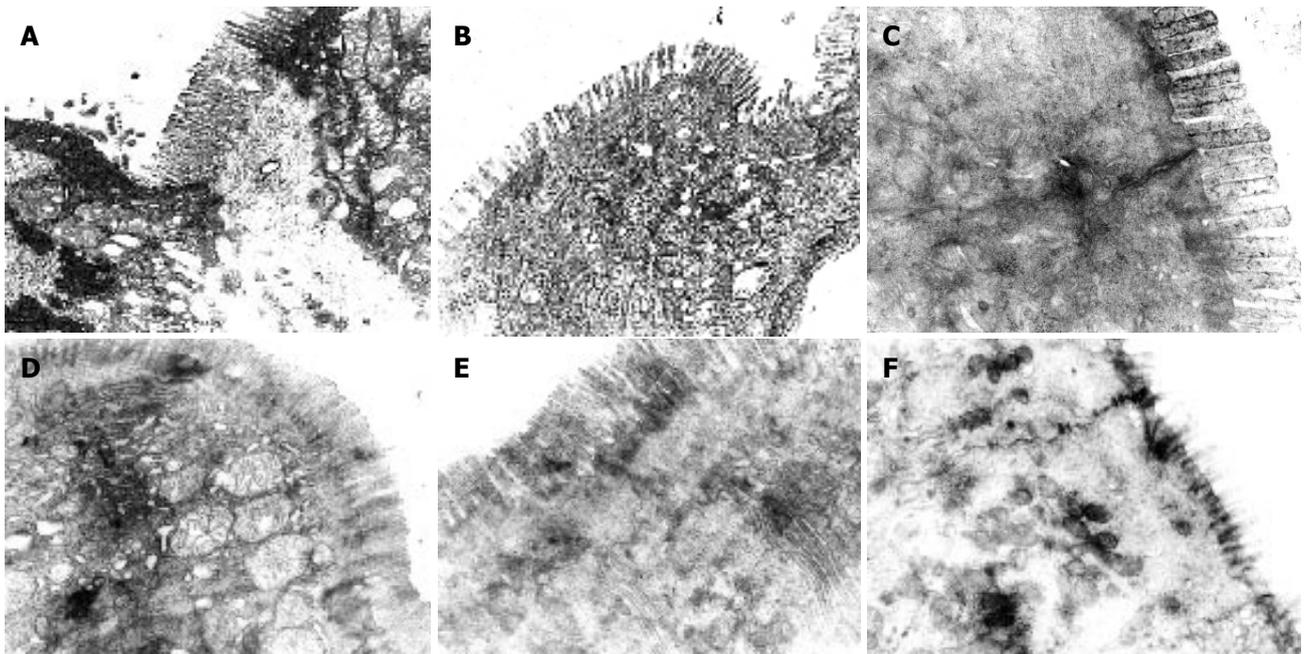


Figure 3 Ultra-structure of tight junction of the intestine and colon. **A:** Tight junction of intestine in PN group (5000 ×); **B:** Tight junction of colon junction in PN group (5000 ×); **C:** Tight junction of intestine in EN group (5000 ×); **D:** Tight junction of the colon in EN group (5000 ×); **E:** Tight junction of intestine in probiotics group (5000 ×); **F:** Tight junction of the colon in probiotics group (5000 ×).

Table 5 Endotoxin and BTR of MLN and remote organs

Group	n	MLN	Liver	Lung	Cava blood	BTR	Endotoxin (EU)
PN	7	6	4	3	4	60.7%	0.403 ± 0.181
EN	7	4	2	2	1	32.1%	0.125 ± 0.040 ^a
Probiotic	7	3	2	1	2	28.6%	0.082 ± 0.029 ^b

^a*P* < 0.05 vs PN group, ^b*P* < 0.01 vs PN group. BTR: Bacterium translocation rate; MLN: Mesenteric lymph node.

subtype of bacterium and only some local bacteria were determined simultaneously by quantitative analysis because of limited experimental condition. Therefore, the common method of anaerobic cultivation could not reflect the whole profiles of gut preponderant bacterial colonies. Fortunately, molecular biology technology (DNA footprint profile of the microbial gene group) has been used in the analysis of the microbiota in recent years, which could not only quantify the amount of the bacteria, but also reflect changes in community profiles to overcome the shortage in this aspect^[8,11]. Our result showed that there were a group of very bright stripes and the number of the stripes in PN group was obviously much more than that in the normal group, EN group and probiotic group, suggesting the gut microflora of the rats only applied with PN were destructed, the species of normal microflora were reduced, and the abnormal intestinal flora increased. The stripes of probiotic group were obviously more than that of the EN group, indicating the species of the rat intestinal microflora increased after adding the probiotics. The 7 stripes in this group were more similar to each other and some specific stripes were identical to the normal group, implying the administration of probiotics was in favor of transferring the rat gut microflora with abdominal infection to the

normal state. On the whole, these results suggest early EN can correct the gut microflora disorder caused by PN, and the balance of gut microflora can be improved after adding the probiotics.

Occludin^[12-16] is one of the major tight junction structural proteins which determine the intestinal selective barrier function. The measurement of the intestinal epithelial transmembrane junction protein (occludin) could reflect the destruction of the gut tight junctions by the pathogen to some degree. Therefore, in our experiment, the expression of occludin on the enterocyte in distal intestine and the colon tissue were detected. The occludin of the PN group was the least in the 3 groups. After EN and probiotics were added, the occludin increased significantly; occludin level in the probiotic group increased more than that in the other two groups. In order to understand the ultrastructure of the tight junction, the samples were primarily observed by electron microscopy. Compared to the PN group, the tight junction form of the small and large intestine was more intact, the arrangement was tighter and the villi kept more integrity in EN group. And the small and large intestinal epithelial forms in probiotic group were more intact and the gut epithelial tight junctions were clearer than other groups. These results suggested the effect of the probiotics on the gut epithelia, especially the large intestine epithelia, was more significant. With improvement of the gut microflora, the expression of the occludin also increased, and the epithelial tight junction appeared more intact by electron microscopy.

There is increasing evidence that probiotic microorganisms can interact with the gut associated lymphoid tissue (GALT) and influence mucosal and systemic immune systems^[17]. We also studied IgA level of the intestinal tissue. Our result using the EN or probiotics

showed that the recovery of the gut IgA expression was more significant than that of the PN group, in the small intestine of EN group and large intestine of probiotic group. Thus using the EN and probiotics could accelerate the expression of occludin and IgA. They could also affect bacterial translocation. After adding the probiotics and/or EN, the death rate, bacterial translocation rates in the blood, mesentery lymph nodes, liver and lung, and the endotoxin level were much lower than the PN group. The death rates and bacterial translocation rates were also decreased in turn.

In conclusion, the mechanism by which EN combined with probiotics improves the gut barrier of the rats with abdominal infection lies in: (1) inhibiting the growth of pathogen and correcting the gut microflora disorder; (2) increasing the expression of occludin and IgA, strengthening the gut epithelial tight junction and the immune state of the local intestine.

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Experimental study of osthole on treatment of hyperlipidemic and alcoholic fatty liver in animals

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Abstract

AIM: To evaluate the effects of osthole on fatty liver, and investigate the possible mechanism.

METHODS: A quail model with hyperlipidemic fatty liver and rat model with alcoholic fatty liver were set up by feeding high fat diet and alcohol, respectively. These experimental animals were then treated with osthole 5-20 mg/kg for 6 wk, respectively. Whereafter, the lipid in serum and hepatic tissue, and coefficient of hepatic weight were measured.

RESULTS: After treatment with osthole the levels of serum total cholesterol (TC), triglyceride (TG), lower density lipoprotein-cholesterol (LDL-C), coefficient of hepatic weight, and the hepatic tissue contents of TC and TG were significantly decreased. The activity of superoxide dismutase (SOD) in liver was improved. In alcohol-induced fatty liver rats, the level of malondialdehyde (MDA) in liver was decreased. In high fat-induced fatty liver quails, glutathione peroxidase (GSH-PX) in liver was significantly improved. The histological evaluation of liver specimens demonstrated that the osthole dramatically decreased lipid accumulation.

CONCLUSION: These results suggested that osthole had therapeutic effects on both alcohol and high fat-induced fatty liver. The mechanism might be associated with its antioxidation.

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Key words: Hyperlipidemic fatty liver; Alcoholic fatty liver; Osthole; Quails; Rats

Song F, Xie ML, Zhu LJ, Zhang KP, Xue J, Gu ZL. Experimental study of osthole on treatment of hyper-

INTRODUCTION

Fatty liver is the first progression of chronic liver diseases, and without therapy, the disease is apt to develop inflammation, necrosis, fibrosis and finally cirrhosis^[1]. There are currently no ideal pharmacological reagents that can prevent or reverse this disease^[1-2].

Osthole is an active constituent isolated from the fruit of *Cnidium monnieri* (L.) Cusson, a Chinese herbal medicine, which has been clinically used as therapy for diseases for many years. It has many anti-inflammatory, anti-osteoporosis and anti-tumour functions, but there is no report about treatment of fatty liver. We found it could decrease the level of serum triglyceride (TG) in ovariectomized rats occasionally, so we begin to study the therapeutic role of osthole for fatty liver.

The aim of our study was to determine whether osthole could reverse steatosis in the rat model of alcohol and quail model of high fat-induced hepatic fat accumulation, and to investigate potential mechanisms involved in this therapeutic effect. Accordingly, we established alcoholic fatty liver rat model and hyperlipidemic fatty liver quail model, and then these animals were treated with osthole.

MATERIALS AND METHODS

Drugs

Osthole was kindly provided by Dr Jia Zhou of Xi'an Green Fount Natural Product Co. Ltd. (China), the purity of the drug was $\geq 95\%$. Lipanthyl was procured from Laboratories Fournier SA (France).

The establishment of model^[3-4]

Male Sprague-Dawley rats weighing 200 ± 20 g were obtained from Animal Breeding Center of Soochow University, and were housed in regular cages situated in an animal room at 22°C. These experimental rats were fed with standard rat diet and allowed to drink water at will. The animal studies were conducted according to the regulations for the use and care of animal experimentation in Soochow University.

The alcoholic fatty liver rat model was induced by

Table 1 Serum levels of TC, TG, HDL-C, LDL-C, FFA and the relative hepatic weight after treatment with osthole for 6 wk in alcohol-induced fatty liver rats ($n = 10$)

Group	TC (mmol/L)	TG (mmol/L)	HDL-C (mmol/L)	LDL-C (mmol/L)	FFA(μ mol/L)	Relative hepatic weight (g/100 g)
Control	2.14 \pm 0.28	0.40 \pm 0.11	1.16 \pm 0.16	0.80 \pm 0.21	429.93 \pm 90.28	2.36 \pm 0.33
Model	2.52 \pm 0.30 ^a	0.51 \pm 0.09 ^a	0.96 \pm 0.24 ^a	1.32 \pm 0.51 ^b	535.04 \pm 105.44 ^a	2.82 \pm 0.28 ^b
Osthole 5 mg/kg	1.72 \pm 0.18 ^d	0.38 \pm 0.08 ^d	0.91 \pm 0.30	0.64 \pm 0.34 ^d	477.37 \pm 109.47	2.30 \pm 0.14 ^d
Osthole 10 mg/kg	1.69 \pm 0.29 ^d	0.36 \pm 0.11 ^d	1.02 \pm 0.17	0.50 \pm 0.17 ^d	473.79 \pm 196.55	2.30 \pm 0.12 ^d
Osthole 20 mg/kg	1.60 \pm 0.36 ^d	0.37 \pm 0.06 ^d	1.05 \pm 0.15	0.43 \pm 0.21 ^d	487.59 \pm 130.79	2.37 \pm 0.19 ^d
Lipanthyl 20 mg/kg	1.82 \pm 0.28 ^d	0.42 \pm 0.09 ^e	1.01 \pm 0.20	0.62 \pm 0.31 ^d	654.74 \pm 136.86 ^c	3.45 \pm 0.18 ^d

^a $P < 0.05$, ^b $P < 0.01$ vs control animal. ^c $P < 0.05$, ^d $P < 0.01$ vs model animal.

feeding 40% alcohol 1 mL/100 g and corn embryo oil 0.4 mL/100 g. Eight wk after the experiment, three rats were killed and livers were taken out for assessment of fatty hepatic development. After the model developed, the rats were randomly divided into 5 groups ($n = 10$ for each group): fatty hepatic model, osthole 5 mg/kg, osthole 10 mg/kg, osthole 20 mg/kg groups, and lipanthyl 20 mg/kg groups respectively. A solvent control group ($n = 10$) was added simultaneously and given 0.5% CMCNa₂ solution. These medications lasted for 6 wk. The rats were then sacrificed, blood sample was obtained and hepatic tissues were collected for measurement of designing parameters

Male quails weighing 120 \pm 5 g were obtained from Animal Breeding Center of Soochow University, they were housed in regular cages situated in an animal room at 22°C, with a 12 h light/12 h dark cycle. The animals were fed with standard quail diet and allowed to drink water at will.

The hyperlipidemic fatty liver quail model was created by feeding a high-fat diet containing standard diet, 10% lard and 2% cholesterol. After 6 wk the fatty liver was generated, these animals were then randomly divided into 5 groups ($n = 10$ for each group): model, osthole 5 mg/kg, osthole 10 mg/kg, osthole 20 mg/kg and lipanthyl 20 mg/kg groups respectively. A solvent control group ($n = 10$) was added as above. These medications lasted for 6 wk. The quails were sacrificed, blood was obtained and hepatic tissues were collected for measurement of designing parameters.

Measurement of serum TC, TG, lower density lipoprotein-cholesterol (LDL-C) and free fatty acid (FFA)

Blood was obtained after 12 h overnight fasting. The assay kits were purchased from Kexin Biology-Technology Company of Shanghai, China. Serum TC, TG, HDL-C and FFA were determined by colorimetric methods according to the procedures provided, LDL-C was obtained by Friedewald calculation, namely, LDL-C = TC - (TG/2.2 + HDL-C).

Measurement of TC and TG in liver

The hepatic TC and TG was extracted from liver tissue using chloroform/methanol mixed solution (1:1, vol: vol), the prepared sample was then centrifuged at 1200 \times g for 10 min, the obtained supernatant was used for measurement of TC and TG according to the colorimetric methods.

Measurement of SOD, MDA, GSH-PX and protein in liver

Liver tissue was taken at the time of animal sacrifice and rapidly put in ice-cold saline; the tissue homogenate (10% wt/vol) was prepared. Contents of SOD, MDA and GSH-PX were determined by colorimetric methods according to the procedure provided, respectively. Protein in liver was also measured by colorimetric method.

Histological observation

Liver specimens were fixed in 10% formaldehyde and embedded in paraffin for HE staining. The degree of fatty degeneration was graded by estimating the proportion of hepatocytes containing fat droplets and expressed as “-, +, ++, +++”. “-” means no fat present, “+” less than 1/3 of the hepatic lobule, “++” 1/3 to 2/3, and “+++” more than 2/3. The histological evaluation of the liver sections was blindly performed.

Statistical analysis

Data are expressed as mean \pm SD, t -test was used for comparisons between groups, χ^2 -test was used for histopathological evaluation. $P \leq 0.05$ was considered statistically significant.

RESULTS

Results from rats

After 6 wk of osthole administration, rat serum TC, TG and LDL-C levels were significantly lower in osthole groups than in model group, the relative hepatic weight in osthole groups was also decreased, but no significant difference was found in serum FFA and HDL-C levels between osthole and model groups (Table 1). The hepatic tissue levels of TC, TG and MDA in osthole groups were significantly lower than those in the model group, and the activity of SOD in osthole groups was increased significantly. The osthole had no obvious effect on GSH-PX activity (Table 2). Histological evaluation of liver specimens demonstrated that osthole could decrease lipid accumulation (Table 3 and Figure 1). The results of the present study suggested osthole could decrease the alcohol-induced liver injury in rats.

The serum levels of TC, TG, LDL-C, FFA and the relative hepatic weight in model group were significantly higher than those in control group, whereas the level of HDL-C was decreased markedly. After treatment with

Table 2 Levels of TC, TG, SOD, MDA and GSH-PX in liver after treatment with osthole for 6 wk in alcohol-induced fatty liver rats ($n = 10$)

Group	TC (mg/g wet tissue)	TG (mg/g wet tissue)	SOD (U/mg prot)	MDA (nmol/mg prot)	GSH-PX (vigor units)
Control	1.94 ± 0.24	15.27 ± 3.35	58.33 ± 4.28	4.14 ± 1.34	174.86 ± 49.56
Model	2.25 ± 0.36 ^a	18.88 ± 3.81 ^a	50.04 ± 8.20 ^a	5.36 ± 0.82 ^a	206.80 ± 66.07
Osthole 5 mg/kg	1.82 ± 0.38 ^d	16.19 ± 4.21	61.11 ± 3.26 ^d	3.90 ± 1.35 ^d	203.80 ± 41.24
Osthole 10 mg/kg	1.80 ± 0.25 ^d	14.25 ± 3.10 ^d	61.45 ± 3.62 ^d	3.74 ± 0.99 ^d	182.30 ± 31.42
Osthole 20 mg/kg	1.76 ± 0.21 ^d	13.67 ± 4.30 ^d	58.91 ± 3.72 ^d	2.88 ± 0.92 ^d	171.89 ± 32.44
Lipanthyl 20 mg/kg	1.61 ± 0.20 ^d	12.84 ± 3.22 ^d	53.80 ± 2.49	2.59 ± 0.54 ^d	200.95 ± 55.26

^a $P < 0.05$, ^b $P < 0.01$ vs control animal. ^c $P < 0.05$, ^d $P < 0.01$ vs model animal.

Table 3 Histopathological changes of hepatic fatty degeneration after treatment with osthole 5-20 mg/kg for 6 wk in alcohol-induced fatty liver rats ($n = 10$)

Degree of fatty degeneration	Control	Model	Osthole			Lipanthyl 20 mg/kg
			5 mg/kg	10 mg/kg	20 mg/kg	
–	9	0	2	2	4	6
+	1	5	7	8	6	4
++	0	5	1	0	0	0
+++	0	0	0	0	0	0
<i>P</i>	< 0.01		< 0.05	< 0.05	< 0.01	< 0.01

P value for medicine-treated or control animal vs model animal.

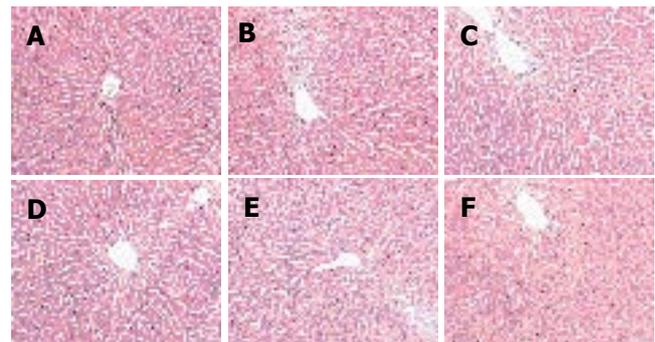


Figure 1 Histopathological changes of rat liver (hematoxylin and eosin staining, 25 ×). No steatosis was observed in control group (A) and the fatty degeneration was seen in the model group (B). Fatty degeneration of the liver was alleviated after treated with osthole 5 mg/kg (C), 10 mg/kg (D), 20 mg/kg (E) and lipanthyl 20 mg/kg (F) for 6 wk.

Table 4 Serum levels of TC, TG, HDL-C, LDL-C, FFA and the relative hepatic weight after treatment with osthole for 6 wk in high fat-induced fatty liver quails ($n = 10$)

Group	TC (mmol/L)	TG (mmol/L)	HDL-C (mmol/L)	LDL-C (mmol/L)	FFA (μmol/L)	Relative hepatic weight (g/100 g)
Control	5.74 ± 0.51	0.68 ± 0.14	5.12 ± 0.48	0.31 ± 0.23	963.21 ± 231.70	1.89 ± 0.16
Model	9.53 ± 3.92 ^b	0.89 ± 0.21 ^a	5.35 ± 0.98	3.78 ± 3.36 ^b	1303.77 ± 372.58 ^a	2.13 ± 0.19 ^b
Osthole 5 mg/kg	7.34 ± 0.88	0.49 ± 0.08 ^d	5.50 ± 0.58	1.62 ± 0.73	938.68 ± 146.77 ^d	1.83 ± 0.11 ^d
Osthole 10 mg/kg	7.32 ± 1.53	0.57 ± 0.16 ^d	5.56 ± 0.93	1.50 ± 1.41	794.34 ± 225.05 ^d	1.83 ± 0.25 ^d
Osthole 20 mg/kg	6.89 ± 0.84 ^c	0.45 ± 0.05 ^d	5.72 ± 0.75	0.98 ± 1.19 ^c	783.96 ± 104.05 ^d	1.78 ± 0.13 ^d
Lipanthyl 20 mg/kg	6.74 ± 0.73 ^c	0.55 ± 0.10 ^d	5.87 ± 0.66	0.62 ± 0.53 ^d	1170.75 ± 425.34	1.76 ± 0.19 ^d

^a $P < 0.05$, ^b $P < 0.01$ vs control animal. ^c $P < 0.05$, ^d $P < 0.01$ vs model animal.

osthole 5-20 mg/kg for 6 wk, serum levels of TC, TG, LDL-C and the relative hepatic weight were decreased by 31.7%-36.5%, 25.4%-29.4%, 51.5%-67.4% and 16.0%-18.4%, respectively. Serum levels of FFA and HDL-C were decreased or increased to some degree, but the effects were not significant compared with those in the model group. In lipanthyl group, serum levels of TC, TG and LDL-C were decreased, but the level of HDL-C was not increased, the level of FFA and the relative hepatic weight were inversely increased.

The tissue levels of TC, TG and MDA in the model group were significantly higher than those in the control group, while the activity of SOD in model group was depressed significantly. Treatment of rats with osthole 5-20 mg/kg for 6 wk, resulted in a significant decline in levels of TC, TG and MDA, and increase in activity of

SOD. In lipanthyl group, the levels of TC, TG and MDA were decreased, but the SOD activity was not increased. There was no obvious effect on GSH-PX activity.

Histological evaluation of liver specimens demonstrated that administration of osthole dramatically decreased lipid accumulation. Thus, our results demonstrated a therapeutic effect of osthole in alcohol-induced fatty liver of the rats.

Results from quails

In quails treated with osthole for 6 wk, serum TC, TG, LDL-C and FFA levels, the relative hepatic weight in osthole groups, particularly in 20 mg/kg group, were significantly lower as compared with model group. No significant difference was observed in HDL-C level between osthole and model groups (Table 4). The hepatic tissue levels of TC and TG in osthole groups

Table 5 Levels of TC, TG, FFA, SOD, MDA and GSH-PX in liver after treatment with osthole for 6 wk in high fat-induced fatty liver quails ($n = 10$)

Group	TC (mg/g wet tissue)	TG (mg/g wet tissue)	FFA (mg/g wet tissue)	SOD (U/mg prot)	MDA (nmol/mg prot)	GSH-PX (vigor units)
Control	2.26 ± 0.56	11.42 ± 4.37	499.14 ± 90.73	368.10 ± 21.60	10.93 ± 2.44	121.53 ± 20.88
Model	6.94 ± 3.25 ^b	17.34 ± 5.90 ^a	418.97 ± 181.75	314.24 ± 17.91 ^b	11.37 ± 1.71	111.23 ± 29.85
Osthole 5 mg/kg	4.32 ± 0.69 ^c	16.05 ± 4.74	508.62 ± 180.05	343.20 ± 33.71 ^c	10.73 ± 2.30	130.00 ± 38.82
Osthole 10 mg/kg	4.05 ± 1.82 ^c	13.48 ± 2.55	478.45 ± 134.74	344.16 ± 10.25 ^d	10.86 ± 3.56	151.40 ± 41.34 ^c
Osthole 20 mg/kg	3.66 ± 1.14 ^d	12.97 ± 2.38 ^c	419.83 ± 106.21	353.70 ± 33.95 ^d	10.73 ± 0.95	174.35 ± 46.43 ^d
Lipanthyl 20 mg/kg	4.05 ± 1.06 ^c	14.87 ± 6.21	731.03 ± 237.13 ^d	357.98 ± 16.08 ^d	11.02 ± 1.90	150.05 ± 46.54 ^c

^c $P < 0.05$, ^d $P < 0.01$ for medicine-treated animal versus model animal.

Table 6 Histopathological changes of hepatic fatty degeneration after treatment with osthole 5-20 mg/kg for 6 wk in high fat-induced fatty liver quails ($n = 10$)

Degree of fatty degeneration	Control	Model	Osthole			Lipanthyl 20 mg/kg
			5 mg/kg	10 mg/kg	20 mg/kg	
—	10	0	3	4	7	5
+	0	4	7	6	3	4
++	0	4	0	0	0	1
+++	0	2	0	0	0	0
<i>P</i>	< 0.01		< 0.01	< 0.01	< 0.01	< 0.01

P value for medicine-treated or control animal *vs* model animal.

were significantly lower than those in model group, and the activities of SOD and GSH-PX were significantly improved. There were no obvious effects on FFA and MDA levels (Table 5). Histological evaluation of liver specimens demonstrated that osthole could decrease lipid accumulation (Table 6 and Figure 2). These results suggested that osthole could decrease the high fat-induced liver injury in quails.

The serum levels of TC, TG, LDL-C, FFA and the relative hepatic weight in model group were significantly higher than those in the control group. Serum levels of TG, FFA and the relative hepatic weight in osthole groups were decreased, while serum levels of TC and LDL-C only in 20 mg/kg group were significantly decreased. In lipanthyl group, serum levels of TC, TG, LDL-C and the relative hepatic weight were also decreased, but serum FFA level was not.

The hepatic tissue levels of TC and TG in model group were significantly higher than those in control group, and the activity of SOD was significantly depressed. Levels of TC and TG in liver in osthole groups were decreased by 37.6%-47.3% and 7.4%-25.2%. Notably, continuous administration of the drug for 6 wk significantly increased the activities of SOD and GSH-PX in hepatic tissue. In lipanthyl group, level of TC was decreased, activities of SOD and GSH-PX in liver were increased, level of FFA was inversely increased, but it had no obvious effects on TG levels.

Histological evaluation of liver specimens demonstrated that administration of osthole dramatically decreased lipid accumulation. Thus, our results demonstrated that osthole possessed a therapeutic effect on high fat-induced fatty liver in quails.

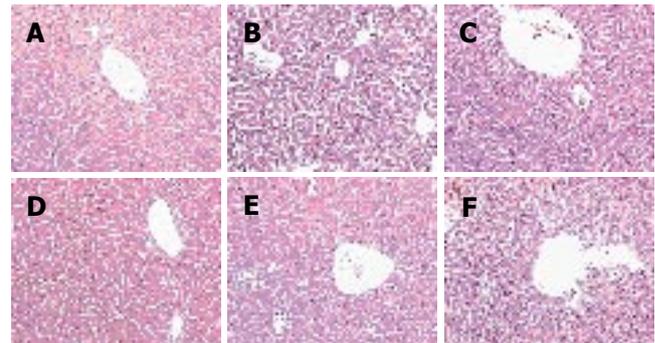


Figure 2 Histopathological changes of quail liver (hematoxylin and eosin staining, 25 ×). No steatosis was observed in control group (A) and the fatty degeneration was seen in the model group (B). Fatty degeneration of liver was alleviated after treated with osthole 5 mg/kg (C), 10 mg/kg (D), 20 mg/kg (E) and lipanthyl 20 mg/kg (F) for 6 wk.

DISCUSSION

Fatty liver can be induced by alcoholic and non-alcoholic aetiologies. It commonly occurs in the general population and has the potential to progress to fibrosis and cirrhosis^[5-6]. The spectrum of pathology is wide, ranging from benign steatosis to cirrhosis, hepatocellular carcinoma and hepatic failure^[6]. The prevalence of hepatic damage in fatty liver is difficult to determine accurately. However, it has been estimated that amongst a population of obese/diabetic individuals approximately 50%-70% will have fatty change, 20%-30% will progress to steatohepatitis/fibrosis and 2%-5% will eventually become cirrhosis^[7-9]. But fatty liver is reversible with abstinence or medicine therapy. To prevent the occurrence of hepatic fibrosis and cirrhosis, treatment of fatty liver is an important step.

Non-alcoholic fatty liver (NAFL) has been extensively reported over the world. In general population studies, screening with ultrasound or CT has shown that a prevalence ranging of NAFL is from 16% to 23%. In liver biopsy studies, the prevalence ranges are 15%-39%^[10-12]. Obesity type 2 diabetes mellitus (DM), female gender and hyperlipidaemia are frequently associated with NAFL^[6,13]. Because the typical dyslipidaemia of NAFL is also characteristic of the commonly occurring insulin resistance syndrome, and there is a strong link between hyperlipidemia and obesity which associate with visceral fat accumulation^[14], so our study emphasized particularly on the hyperlipidemic fatty liver.

Alcohol is also a major cause of fatty liver. Alcoholic

fatty liver (AFL) has a common pathophysiology with NAFL^[15]. At least 80% of heavy drinkers develop fatty liver. It is reversible with abstinence, whereas it may progress to cirrhosis without abstinence.

At present the treatment for fatty liver mainly includes dietary intervention, treatment of associated insulin resistance, lipid-lowering medications, antioxidants and ursodeoxycholic acid and so on. An ideal and effective drug has not been found to prevent and treat this disease^[6].

Our experimental results show that after treatment with osthole for 6 wk, the levels of serum TC, TG, LDL-C, the relative hepatic weight and the hepatic tissue contents of TC and TG were decreased significantly in alcohol-induced fatty liver rats or in high fat-induced fatty liver quails. The histological evaluation of liver specimens demonstrated that osthole dramatically decreased lipid accumulation. These results suggest that osthole has a new function to protect the liver from fat accumulation. It decreased high fat-induced liver injury in quails and alcohol-induced liver injury in rats.

To develop fatty liver, it has been recently suggested that one prerequisite condition should exist, i.e., a source of oxidative stress capable of initiating significant lipid peroxidation^[16]. Both animal data and human studies have shown a link between fatty liver and oxidative stress and lipid peroxidation. Mitochondria are thought to be the source of the reactive oxygen species (ROS) leading to lipid peroxidation. The increased hepatic influx of FFA that results from the reduced ability of insulin to suppress lipolysis is thought to increase the rate of mitochondrial [beta] oxidation and produce the ROS. There are other potential sources of oxidative stress including the cytochrome P450 enzymes (CYP2E1 and CYP3A4) and increased hepatic iron content. Chronic alcohol exposure may also result in oxidant production^[16].

Our experimental results showed in the alcohol-induced fatty liver test that administration of osthole promoted SOD production, depressed MDA production, and lightened the degree of steatosis in liver tissue. In the high fat-induced fatty liver test, administration of osthole had same effects as in the alcohol-induced fatty liver test. Furthermore, osthole also promoted GSH-PX production in liver tissue. These results demonstrated that the therapeutic effect of osthole on fatty liver was partly due to its antioxidation against ROS. From the findings of

present studies, we suggested that the mechanism of fatty liver generation was related to the extra production of ROS.

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BASIC RESEARCH

Role of neuronal nitric oxide synthase and inducible nitric oxide synthase in intestinal injury in neonatal rats

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Abstract

AIM: To investigate the dynamic change and role of neuronal nitric oxide synthase (nNOS) and inducible nitric oxide synthase (iNOS) in neonatal rat with intestinal injury and to define whether necrotizing enterocolitis (NEC) is associated with the levels of nitric oxide synthase (NOS) in the mucosa of the affected intestine tissue.

METHODS: Wistar rats less than 24 h in age received an intraperitoneal injection with 5 mg/kg lipopolysaccharide (LPS). Ileum tissues were collected at 1, 3, 6, 12 and 24 h following LPS challenge for histological evaluation of NEC and for measurements of nNOS and iNOS. The correlation between the degree of intestinal injury and levels of NOS was determined.

RESULTS: The LPS-injected pups showed a significant increase in injury scores versus the control. The expression of nNOS protein and mRNA was diminished after LPS injection. There was a negative significant correlation between the nNOS protein and the grade of median intestinal injury within 24 h. The expression of iNOS protein and mRNA was significantly increased in the peak of intestinal injury.

CONCLUSION: nNOS and iNOS play different roles in LPS-induced intestinal injury. Caution should be exerted concerning potential therapeutic uses of NOS inhibitors in NEC.

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Key words: Neuronal nitric oxide synthase; Inducible nitric oxide synthase; Necrotizing enterocolitis; Rat; Newborn

INTRODUCTION

Necrotizing enterocolitis (NEC) is a common and devastating gastrointestinal condition of neonatal infants^[1]. In spite of extensive epidemiological, clinical, and basic research, the pathogenesis of NEC remains elusive, and there is no effective preventative treatment for this disease. Several lines of evidence suggest that neonatal risk factors of prematurity, intestinal asphyxia/ischemia, formula feeding and bacterial colonization all contribute to the occurrence of the disease^[1-4]. These components probably act in concert to upset an already immature and delicate intestinal mucosal barrier^[5].

Nitric oxide (NO) is a weak radical produced from L-arginine via the nitric oxide synthase (NOS) isoforms: the constitutive neuronal NOS (type I, nNOS), the inducible NOS (type II, iNOS), and the endothelial NOS (type III, eNOS). The endogenous NO is important in defending against bowel injury and mortality^[3,6]. Previous studies suggest an important role for iNOS in the pathogenesis of NEC. These include the terminal ileum from rats with abnormal histology demonstrated increased iNOS expression^[4,7]. Not all studies, however, have found inhibition of NO production to be beneficial in septic animals or animals challenged with endotoxin. For example, Park *et al*^[8] found that mice receiving a lethal dose of endotoxin and L-NMMA died earlier and had increased lung, liver, and kidney tissue damage than mice receiving endotoxin only. A recent study^[9] reported that iNOS knockout mice exhibited an aggravated intestinal injury and inflammation with enhanced neutrophil infiltration in response to acetic acid instillation. Qu *et al*^[10] reported that constitutive NOS is the predominant NOS in the intestine and its activity is inversely correlated with the level of tissue injury. But these experiments were not conducted in newborn animals.

Therefore, the purpose of the present study was to investigate the dynamic change and role of nNOS and iNOS in neonatal rat with LPS-induced intestinal injury and to define whether NEC is associated with the levels of NOS in the mucosa of the affected intestine tissue.

MATERIALS AND METHODS

Animal model

Wistar rats less than 24 h in age (mean weight, 6.24 ± 0.81 g) were given an intraperitoneal (IP) injection of 5 mg/kg *E coli* O55:B5 endotoxin (LPS; Sigma Chemical Co., St. Louis, Mo, USA) or similar volume of saline^[11-13]. All pups were killed respectively at 1, 3, 6, 12 and 24 h after receiving LPS IP ($n = 8$). Control pups ($n = 8$) were killed at 1 h after saline IP. The pups which died before collection of the specimens were excluded from the study.

Specimens collection

All surviving animals were killed via decapitation. The gastrointestinal tract was carefully removed. The small intestine was then divided into two halves: jejunum and ileum. A 3 cm segment of distal ileum 4 cm proximal to the ileocecal valve from each animal was cut, and fixed for histological evaluation of NEC and immunohistochemical analysis. The rest of the ileum was snap frozen at -80°C for measurements of mRNA.

Experimental methods and analysis marker

NEC evaluation: The segment of distal ileum was harvested, fixed in 4% paraformaldehyde, embedded in paraffin, microtome-sectioned at 5 μm , and counterstained with hematoxylin and eosin for histological evaluation of intestinal injury. Histological changes in the ileum were scored by a blinded evaluator and were assigned a necrotizing enterocolitis (NEC) score on a scale of 0 to 4 as follows: 0 = normal, intact villous epithelium with normal histology; 1 = mild villous edema, with epithelial sloughing confined to the tips of the villi; 2 = mild midvillous necrosis; 3 = moderate midvillous necrosis, with crypts still readily detectable; and 4 = severe necrosis of entire villi with complete absence of epithelial structures^[13,14].

Immunohistochemistry: The localization of nNOS and iNOS was performed by the SABC immunohistochemical technique. The paraffin slices were routinely dewaxed and the antigens were retrieved by microwave. Intrinsic peroxidases were inactivated by 3% hydrogen peroxide solution. The slices were blocked at room temperature for 10 min by normal goat serum. Primary antibody (rabbit anti-rat nNOS and iNOS antibody) in 1:200 dilution was added and left overnight at 4°C . The slices were then washed with PBS, and after biotinylated goat anti-rabbit IgG was added they were left at 37°C for 35 min. They were then washed with phosphate-buffered saline (PBS) and avidin-peroxidase conjugates were added and they were then left at 37°C for 35 min. They were then washed with PBS 3 times (5 min each time) and DAB solution was added for staining at room temperature. The reaction time (5 min) was controlled under the microscope. Slices were then routinely washed and gently restained with hematoxylin and sealed. (The above-mentioned antibody and reagent were purchased from Beijing Zhongshan Biotechnology Co., Ltd). For the negative control group, PBS was used instead of rat nNOS and iNOS antibody. The cells with yellow-brown particle deposition in cytoplasm were judged to be positive.

Five clearly dyed slices at each time point were taken randomly. Then 5 random fields for every slice were selected under microscope ($\times 40$), and the windows' square was fixed. The contents of nNOS and iNOS were semi-quantitatively measured through optical density average by Meta Morph and computer image process software.

RT-PCR for nNOS, iNOS and β -actin: Total RNA was extracted using the BiotragentsTM reagent (Sino-American Biotechnology Co., Luoyang, China) and 2 μL RNAs were used to synthesize cDNA in the presence of an oligo dT 15-primer, RNase inhibitor and the AMV reverse transcriptase in a final volume of 20 μL . Sequence-specific oligonucleotide primers (Bioasia Biotechnology Co., Ltd, Shanghai, China) were designed according to rat podocin, nNOS, sense: 5'-GAA TAC CAG CCT GAT CCA TGG AA-3', antisense: 5'-TCC TCC AGG AGG GTG TCC ACC GCA TG-3'; iNOS, sense: 5'-ATC CCG AAA CGC TAC ACT T-3', antisense: 5'-TCT GGC GAA GAA CAA TCC-3'; β -actin, sense: 5'-CAC CCT GTG CTG CTC ACC GAG GCC-3', antisense: 5'-CCA CAC AGA TGA CTT GCG CTC AGG-3'. The expected size of amplification was 602 bp for nNOS, 690 bp for iNOS and 314 bp for β -actin. PCR was performed in a 25 μL reaction system which contained 3 μL cDNA, 17.1 μL ddH₂O, 10 \times PCR buffer 2.5 μL , 2.5 mmol/L dNTPs 2 μL , Taq DNA polymerase 0.2 μL (TaKaRa Biotechnology Co., Ltd, Dalian, China), 0.1 μL of each primer. Amplification cycles of nNOS were 94°C for 3 min, followed by 30 cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 1 min and terminated by a final extension of 72°C for 10 min. Amplification cycles of iNOS were 95°C for 1.5 min, followed by 45 cycles at 94°C for 45 s, 55°C for 45 s and 72°C for 1.5 min and terminated by a final extension of 72°C for 10 min.

The PCR products were subjected to electrophoresis with 2% agarose gel and stained with ethidium bromide. The band intensity was determined by gel image analysis system (Kodak 1D, USA). Relative concentrations of mRNA were normalized for β -actin. Expression levels of nNOS mRNA and iNOS mRNA were calculated by dividing the intensity of the internal control, β -actin.

Statistical analysis

Software SPSS 11.0 for Windows was used in all statistical tests. Comparisons between the groups were calculated using one-way analysis of variance (ANOVA), and all data are expressed as mean \pm SD. When P was less than 0.05, the difference was considered statistically significant. The degree of correlation was described using the Spearman's rank-correlation test.

RESULTS

Incidence and severity of NEC

There was a normal, intact villous epithelium with normal histology of ileum tissue in the control group. Mild villous edema, with epithelial sloughing confined to the tips of the villi could be seen at 1 h following LPS injection, and midvillous necrosis was aggravated from top to the lower part and the amount of the midvillous necrosis in neonate rats was increased with time. The most deteriorating

Table 1 Scores of lesion on distal ileum morphology of neonatal rats (mean \pm SD, $n = 8$)

Score	Control group	LPS group				
		1 h	3 h	6 h	12 h	24 h
	0.12 \pm 0.17	1.28 \pm 0.62 ^b	1.75 \pm 0.74 ^b	1.98 \pm 0.75 ^b	2.85 \pm 0.41 ^b	2.35 \pm 0.63 ^b
≥ 2 (n)	0	1	4	5	7	6

^a $P < 0.05$, ^b $P < 0.01$ vs control group.

Table 2 Optical density of nNOS and iNOS in ileum of neonatal rats (mean \pm SD, $n = 5$)

	Control group	LPS group				
		1 h	3 h	6 h	12 h	24 h
nNOS	0.3987 \pm 0.0020	0.3975 \pm 0.0020	0.3965 \pm 0.0020	0.3896 \pm 0.0021 ^b	0.3806 \pm 0.0031 ^c	0.3836 \pm 0.0027 ^c
iNOS	0.3806 \pm 0.0080	0.3832 \pm 0.0060	0.3869 \pm 0.0050	0.3870 \pm 0.0070	0.3968 \pm 0.0101 ^a	0.3812 \pm 0.0070

^a $P < 0.05$, ^b $P < 0.01$, ^c $P < 0.001$ vs control group.

Table 3 nNOS mRNA and iNOS mRNA of ileum of neonatal rats (mean \pm SD, $n = 8$)

	Control group	LPS group				
		1 h	3 h	6 h	12 h	24 h
nNOS	0.88 \pm 0.23	0.72 \pm 0.34	0.65 \pm 0.32	0.39 \pm 0.23 ^b	0.47 \pm 0.31 ^a	0.64 \pm 0.35
iNOS	0.70 \pm 0.17	0.73 \pm 0.19	0.75 \pm 0.21	0.97 \pm 0.23 ^a	0.87 \pm 0.29 ^a	0.78 \pm 0.22

^a $P < 0.05$, ^b $P < 0.01$ vs control group.

change was at 12 h and the incidence of NEC was 87.5% (7/8). There was severe necrosis of entire villi with complete absence of epithelial structures. The injury score of ileum tissue in the LPS group was also significantly increased than the control group pups ($P < 0.01$) (Table 1).

Expression of nNOS and iNOS proteins

The positive expression of nNOS was mainly shown in the nerve plexus and nerve fiber of the small intestinal wall. The myenteric nerve plexus had relatively higher levels of nNOS expression in comparison with the submucous nerve plexus. The positive expression of nNOS was decreased after LPS injection, and was significantly different at 6-24 h following LPS treatment ($P < 0.01$). The lowest level of its expression was at 12 h ($P < 0.001$) (Table 2). There was a negative significant correlation between the nNOS and the grade of median intestinal injury within 24 h ($\gamma = -1.000$, $P < 0.01$). The iNOS protein was localized almost exclusively to the epithelial cells (enterocytes) in the surface villi, with some staining detected in the lamina propria. The positive expression of iNOS in LPS group only increased at 12 h ($P < 0.05$) (Table 2). There was no a significant correlation between the nNOS and the grade of median intestinal injury within 24 h ($\gamma = 0.400$, $P > 0.05$).

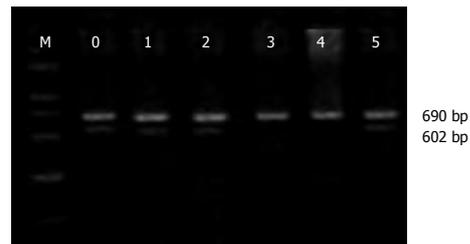


Figure 1 Gene expression of nNOS in ileum of neonatal rats. 2% ethidium bromide stained agarose gel molecular marker (Kodak 1D) was used as an indicator. M: DNA Marker DL2000; 0: Control group; 1: LPS 1 h; 2: LPS 3 h; 3: LPS 6 h; 4: LPS 12 h; 5: LPS 24 h.

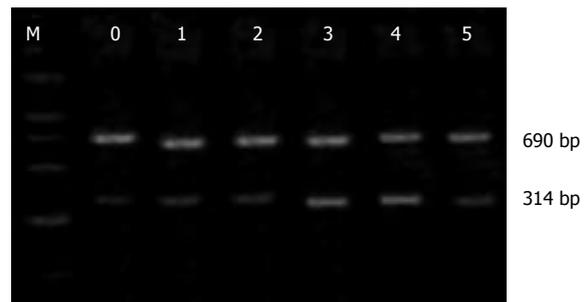


Figure 2 Gene expression of iNOS in ileum of neonatal rats. 2% ethidium bromide stained agarose gel molecular marker (Kodak 1D) was used as an indicator. M: DNA marker DL2000; 0: Control group; 1: LPS 1 h; 2: LPS 3 h; 3: LPS 6 h; 4: LPS 12 h; 5: LPS 24 h.

Expression of nNOS mRNA and iNOS mRNA

Expression of nNOS mRNA in ileal tissue was gradually diminished after LPS injection. The level of its expression was significantly lower at 6 and 12 h ($P < 0.05$) (Table 3, Figure 1). The expression of iNOS mRNA was significantly increased at 6, 12 h following LPS treatment ($P < 0.05$) (Table 3, Figure 2).

DISCUSSION

In the present study, we detected substantially lower levels of nNOS in ileal tissue with complicated intestinal injury when compared with a cohort of control rats. These findings demonstrated a potential role for a deficiency of nNOS in the progression of NEC. There was a significant negative correlation between the levels of nNOS and the degree of intestinal injury. These results showed that decreased levels of nNOS of the ileum result in a dramatic increase of the incidence and severity of NEC. The iNOS

mRNA and protein expression was significantly increased during the peak of intestinal injury. The production of NO may lead to consequent enterocyte necrosis, gut barrier failure with further bacterial invasion, and damage to the intestinal wall.

NEC is the most frequent and most lethal disease that affects the gastrointestinal tract of premature infants in neonatal intensive care units, with reported mortality of 10%-30%^[15,16]. The reasons for a predilection for prematurity are unclear, but an immature mucosal barrier and immune response likely contribute to the premature neonates' susceptibility^[3,5,17]. Perinatal insults that impair mesenteric circulation may therefore induce intestinal mucosal injury and permit local intestinal microbial flora to breach the mucosal barrier. This process, in turn, initiates an inflammatory cascade leading to NEC^[15,18,19].

Over the past decade, an abundance of research has been directed toward the role of NO in intestinal inflammation. NO can exert both beneficial and deleterious effects and that NOS exists in three distinct isoforms: constitutively expressed nNOS and eNOS or iNOS^[6]. More than 90% of the total NOS in the small intestine is nNOS. Although iNOS is constitutively present, it accounts for less than 10% of the total NOS activity, and eNOS is barely detectable in the intestine^[3,10,20]. iNOS is induced by cytokines or endotoxin and produces large amounts of NO.

The main function of nNOS in the intestine is generally believed to be mediation of the neuronal signal transmission in the NANC components of the nervous system and regulating gut motility. Since eNOS accounts for only a very small part of intestinal NOS activity, it is possible that nNOS in the intestine also functions as the protective eNOS^[21].

In the intestine, constitutive NOS may have important physiological functions. The relatively small amounts of NO production by constitutive NOS have been implicated as scavengers of oxidants that may thus protect an organ in the early stages of injury. For example, NO may protect the gastrointestinal mucosa from a variety of stimuli including endotoxin by maintaining mucosal perfusion, inhibiting leukocyte and platelet adhesion to the endothelium, preventing mast cell activation, and acting as an antioxidant^[6,22-24]. Our present study showed that the positive expression of nNOS was decreased after LPS injection, which was significantly different at 6-24 h following LPS challenge. The lowest level of its expression was at 12 h. There was a negative significant correlation between the nNOS and the grade of median intestinal injury within 24 h. The expression of nNOS mRNA in the ileum tissue was gradually diminished after LPS injection. LPS may downregulate the expression of nNOS mRNA in damaged ileum and may lead to decreased NO production, which results in impairment of endogenous protective mechanisms in the mucosal barrier^[3,6,25]. It may be one of the most important mechanisms resulting in NEC.

The overproduction of NO by iNOS can interact with other reactive oxygen metabolites, such as superoxide, resulting in the propagation of the highly reactive species, peroxynitrite^[26,27]. This potent oxidizing agent can initiate lipid peroxidation and thus produce extreme

gastrointestinal mucosal damage and injury^[4,9,28]. However, recent studies have challenged this simple paradigm providing evidence that iNOS could also be protective against inflammatory response under certain circumstances^[9]. McCafferty *et al.*^[29] reported that iNOS deficiency was not important in IL-10 deficient mice with spontaneous chronic intestinal inflammation. Our data showed that the positive expression of iNOS protein only increased at 12 h and the expression of iNOS mRNA was significantly increased at 6, 12 h following LPS injection. The iNOS mRNA and protein expression was significantly increased in the peak of intestinal injury. The production of NO may lead to consequent enterocyte necrosis, gut barrier failure with further bacterial invasion, and damage to the intestinal wall. However, the present study failed to show a clear correlation between the nNOS protein and the grade of median intestinal injury.

In conclusion, the nNOS and iNOS play different roles in neonatal rats with LPS-induced intestinal injury. Caution should be exerted concerning potential therapeutic uses of NOS inhibitors on NEC.

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Effect of insulin-sensitizing agents in combination with ezetimibe, and valsartan in rats with non-alcoholic fatty liver disease

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Abstract

AIM: To assess whether treatment with insulin-sensitizing agents (ISAs) in combination with ezetimibe and valsartan have greater effect on hepatic fat content and lipid peroxidation compared to monotherapy in the methionine choline-deficient diet (MCDD) rat model of non-alcoholic fatty liver disease (NAFLD).

METHODS: Rats ($n = 6$ per group) were treated with different drugs, including MCDD only, MCDD diet with either metformin (200 mg/kg), rosiglitazone (3 mg/kg), metformin plus rosiglitazone (M+R), ezetimibe (2 mg/kg), valsartan (2 mg/kg), or combination of all drugs for a total of 15 wk. Liver histology, lipids, parameters of oxidative stress and TNF-alpha were measured.

RESULTS: Fatty liver (FL) rats demonstrated severe hepatic fatty infiltration (> 91% fat), with an increase in hepatic TG (+1263%, $P < 0.001$), hepatic cholesterol (+245%, $P < 0.03$), hepatic MDA levels (+225%, $P < 0.001$), serum TNF-alpha (17.8 ± 10 vs 7.8 ± 0.0 , $P < 0.001$), but a decrease in hepatic alpha tocopherol (-74%, $P < 0.001$) as compared to the control rats. Combination therapy with all drugs produced a significant decrease in liver steatosis (-54%), hepatic TG (-64%), hepatic cholesterol (-31%) and hepatic MDA (-70%), but increased hepatic alpha tocopherol (+443%) as compared to FL rats. Combination therapy with ISA alone produced a smaller decrease in liver steatosis (-32% vs -54%, $P < 0.001$) and in hepatic MDA levels (-55% vs -70%, $P < 0.01$), but a similar decrease in hepatic lipids when compared with the all drugs combination. TNF-alpha levels decreased significantly in all treatment

groups except in ISA group.

CONCLUSION: Combination therapies have a greater effect on liver fat content as compared to monotherapy. Rosiglitazone appears to improve hepatic steatosis to a greater extent than metformin.

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Key words: Fatty liver; Rosiglitazone; Metformin; Ezetimibe; Valsartan; Methionine choline-deficient diet; Insulin resistance

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INTRODUCTION

The clinical implications of fat accumulation in the liver are derived mostly from its occurrence in the general population and it has potential to progress to fibrosis, cirrhosis and hepatocellular carcinoma^[1-3]. Non-alcoholic fatty liver disease (NAFLD) is a component of the metabolic syndrome with a clinical spectrum ranging from simple FL to steatohepatitis, bridging fibrosis and cirrhosis^[4,5]. Obesity and diabetes type 2 are considered the most powerful predisposing risk factors for the development of more severe manifestations of NAFLD^[5,6]. The primary event of non-alcoholic steatohepatitis (NASH) is the accumulation of triglycerides in hepatocytes which seems to be determined by insulin resistance^[3]. These fats stem mainly from increased splanchnic lipolysis of visceral and subcutaneous abdominal fat and from continuous delivery of free fatty acids to the liver after ingestion of fatty foods, both of which increase hepatic insulin resistance^[7]. The secondary event is hepatocellular injury which includes factors, such as oxidative stress, pro-inflammatory cytokines, mitochondrial dysfunction, iron overload, bacterial overgrowth and genetic predisposition^[8]. A recent study by Wanless and Shiota^[9] describes an inflammatory injury to hepatic veins due to release of fat followed by venous obstruction with secondary collapse

and ultimately bridging fibrosis and progressing from fatty liver to NASH to cirrhosis. More recently, deregulated adipocytokines such as adiponectin, interleukin-6 and TNF- α have been examined as causative candidates of insulin resistance^[7,10] and it was reported that oxidative stress in accumulated fat causes deregulated adipocytokine production^[11].

The methionine choline-deficient diet (MCDD) model of fatty liver in rats is characterized by increased insulin resistance, hypertriglyceridemia, and increased lipid peroxidation^[12,13]. Moreover, it has been shown that in the MCDD model of steatohepatitis, there is down-regulation of insulin signaling with decreased phosphorylation of IRS-1, IRS-2 and Akt and increased oxidative stress with over-expression of CYP2E1^[14]. In light of these findings, it seems possible that NAFLD can be successfully treated by the reduction of fat absorption and delivery, oxidative stress and systemic inflammation, as well as reduction of insulin resistance. To date, no consistently effective therapy for FL disease has been identified. The need to use drugs with different and complementary mechanisms of action frequently arises in daily clinical practice. Possible therapeutic agents for fat-induced hepatic insulin resistance include metformin, which acts by decreasing gluconeogenesis and by enhancing peripheral glucose uptake^[15] and thiazolidinediones, a peroxisome proliferator-activated receptor-gamma (PPAR- γ) agonist that acts by increasing insulin sensitivity in adipose tissues, shifting fat away from the liver into fat stores (adipocytes)^[16]. The combination of metformin and rosiglitazone may be complementary. Other therapeutic agents for insulin resistance include valsartan, an angiotensin 11 type-1 receptor blocker which stimulates the insulin signaling cascade and enhances glucose transporter type 4 (GLUT 4) translocation^[17] and ezetimibe, the cholesterol absorption inhibitor which improves insulin resistance by decreasing low-density lipoprotein (LDL) tendency to peroxidation^[18]. In the present study, we aimed to examine the impact of these different therapeutic interventions, alone or in combination, on hepatic fat content, hepatic lipid composition and parameters of oxidative stress, anti-oxidant capability and systemic inflammation in FL rats fed by MCDD.

MATERIALS AND METHODS

Animal and protocol

This study consisted sixty male Sprague Dawley rats (Harlan Laboratories Limited, Jerusalem, Israel), weighing 200-280 g. Rats were housed in regular cages situated in an animal room at 22°C, maintained on standard rat chow diet (Koffolk, Tel Aviv, Israel) and given tap water to drink *ad libitum*. All animal studies were conducted according to the regulations for the use and care of experimental animals and treatment groups.

FL was induced in rats fed by MCDD diet for 9 wk as previously described^[19,20]. The rats were randomly divided into nine groups. Group 1 comprised of 6 rats serving as control group and was maintained on standard chow diet (control liver, CL). Group 2 (FL group or NAFLD

group) was given MCDD only for 9 wk (MCDD Harlan Teeklad, Madison, WI). The following groups were given various pharmaceutical interventions for additional 6 wk: Group 3 received MCDD diet with metformin (200 mg/kg); Group 4 received MCDD diet with rosiglitazone (3 mg/kg); Group 5 was fed MCDD diet with metformin + rosiglitazone; Group 6 received MCDD diet with ezetimibe (2 mg/kg); Group 7 were fed MCDD diet with valsartan (2 mg/kg); Group 8 received MCDD diet with metformin + rosiglitazone + valsartan; and Group 9 received MCDD diet with metformin + rosiglitazone + valsartan + ezetimibe. All treatment groups were treated for a total of 15 wk (9 + 6 wk). The dosage selected for each intervention was based on the results of previous studies using these agents in various liver diseases and the dose of each drug was controlled by semi-quantitative analysis^[16-18]. Diets were supplied in pellet form. The medication was given with food and in drinking water. Simply the MCD diet was supplemented with the respective drug and not with a semi-purified diet. All the drugs were water soluble and there was no need for additional solvents. The drugs were monitored daily and were supplied by the local pharmacy of the Sieff Hospital, Safed, Israel and not by pharmaceutical companies. Rats were then sacrificed and liver histology, hepatic and plasma lipid content, parameters of oxidative stress, and TNF- α were measured.

Biochemistry

Serum triglyceride (TG) concentration was measured with an automated analyzer (Olympus AU2700; Hamburg, Germany). Additional biochemical parameters, such as serum total cholesterol (TC) and glucose, were measured using an automatic biochemical analytical system. Alanine (ALT) transaminase activities were performed spectrophotometrically using a commercially available kit test (ALT Reagents, Sigma-Aldrich). Tumor necrosis factor alpha (TNF- α) was assayed by rat-specific kit (R&D Systems). Insulin was assayed by a rat-specific RIA kit (Incstar, Stillwater, MI). Blood insulin resistance was estimated using the homeostasis model assessment (HOMA-IR) derived from the following equation: IR = (fasting plasma glucose level mg% \times 0.055) \times (fasting plasma insulin level mU/L) /22.5). This insulin resistance reflects both peripheral and hepatic insulin resistance^[21].

Determination of hepatic lipid composition

Total hepatic lipids were extracted from freeze-dried samples by chloroform: methanol (2:1) and measured as previously described by Folch *et al*^[22]. Triglycerides, TC and phospholipids in hepatic tissue extract were determined enzymatically in an auto analyzer (Hitachi 736, Tokyo, Japan) using commercial kits (Triglycerides, Infinity Cholesterol Reagent and Reagents for inorganic phosphorous assay, Sigma-Aldrich) as previously described^[23-26].

Determination of hepatic pro-oxidant and anti-oxidants

Tissue tocopherol-alpha was determined following the methods described by Gowenlock *et al*^[27]. Hepatic maleic dialdehyde (MDA) level was determined as previously described by Yagi *et al*^[28].

Histology

A 5- μ m thick section of liver tissue was cut from the center of each hepatic lobe and fixed in 40 g/L buffered formaldehyde, processed by standard techniques and embedded in paraffin. The sections were stained with hematoxylin-eosin. Fat extension, necroinflammatory grade and stage of fibrosis were assessed as previously described by Brunt *et al.*^[29]. Steatosis was assessed by a morphological semiquantitative approach and graded as follows: mild = 5%-30%, moderate = 30%-60%, and severe > 60% of hepatocytes affected. The specimens were also examined for histological features of Mallory's bodies, ballooning degeneration, acidophilic necrosis, sinusoidal fibrosis and polymorph nuclear infiltrates. The histological evaluation of the liver sections was performed blindly.

Statistical analysis

Results are expressed as mean \pm SE. Analysis of variance was used to compare the means of multiple groups, followed by the Newman-Keuls test to determine statistical significance between two groups. When the data were not normally distributed, the Kruskal-Wallis test was performed to compare the means of multiple groups, followed by Mann-Whitney test. Correlation analysis was performed using spearman rank correlation. The statistical comparisons were performed using the unit values rather than percentages. $P < 0.05$ was considered statistically significant. The statistical analysis was performed using the Winstat program for windows (Kalmia, MA).

RESULTS

Effect of methionine choline-deficient diet

The methionine choline FL model used in this experimental study showed features of the fatty liver observed in humans, including hypertriglyceridemia, increased oxidative stress, increased insulin resistance and increased ALT and TNF- α levels (Tables 1 and 2). FL induced by the MCDD diet had a 68% increase in the liver weight/rat weight ratio when compared to CL (Figure 1, $P < 0.02$). Hepatic TG and hepatic cholesterol concentrations were significantly higher in the rats fed MCDD diet than that in the control rats (+1263% increase, $P < 0.001$ and +245% increase, $P < 0.03$, respectively). Triglycerides represented the highest proportion of lipid components of the fat vesicles. FL rats showed a 100% increase in plasma triglycerides and 157% increase in HOMA-insulin resistance index ($P < 0.05$ and $P < 0.001$, respectively). FL rats had significantly lower concentrations of alpha-tocopherol when compared with CL group ($P < 0.03$). The hepatic levels of MDA were significantly greater and hepatic alpha-tocopherol/MDA ratio was significantly lower in FL group as compared with CL group (2.01 ± 0.04 vs 0.6 ± 0.1 ; $P < 0.03$ and 0.03 vs 0.05 , $P < 0.03$, respectively). Specimens from the rats fed with methionine choline-deficient diet showed massive fatty infiltration (> 91%), predominantly macrovesicular. Features of steatohepatitis, including ballooning degeneration and pericellular fibrosis, were seen mildly but no pronounced fibrosis.

Table 1 Liver-related parameters, plasma biochemistry and cytokines in rats fed with methionine choline-deficient diet (MCDD) or choline-supplemented diet (Chow diet) (mean \pm SD, $n = 6$)

Parameters	CL	FL	<i>P</i> values
	Chow diet	MCDD diet	
Baseline rat mass (g)	227 \pm 7	226 \pm 6	0.5
Final rat mass (g)	406 \pm 19	257 \pm 12	0.03
Final liver mass (g)	10.2 \pm 0.3	10.8 \pm 0.8	0.4
Liver/body mass ratio	0.025 \pm 0.003	0.042 \pm 0.003	0.02
% change		68	
Liver triglycerides (mol/g)	1.6 \pm 0.3	21.8 \pm 1.0	< 0.001
% change		1263	
Liver cholesterol (mol/g)	0.5 \pm 0.0	1.6 \pm 0.2	< 0.03
% change		245	
Serum HOMA-IRI	7 \pm 0.3	18 \pm 2.0	< 0.001
Liver tocopherol/MDA ratio	0.05	0.003	< 0.03
FL infiltration (%)	0	91 \pm 5	< 0.001
ALT (nkat/L)	667 \pm 83	800 \pm 33	< 0.05

CL: Control liver; FL: Fatty liver; HOMA-IRI: Insulin resistance index; MDA: Malondialdehyde.

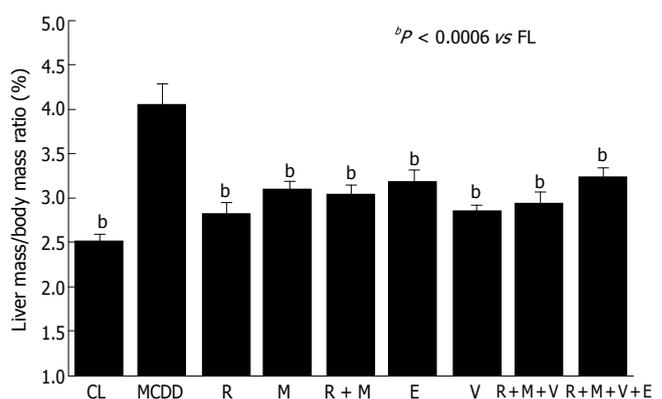


Figure 1 Effect of different pharmacological interventions on liver mass/body mass ratio.

Effect of pharmacologic intervention

All the drug regimens included in this study significantly decreased the liver weight/body weight ratio as compared to FL rats fed with the methionine choline-deficient diet alone (range 24%-33%, Figure 1)

Effect of insulin-sensitizing agents

Rosiglitazone improved insulin resistance (-39%, $P < 0.03$) and reduced hepatic and plasma triglycerides by -54% ($P < 0.01$) and -59% ($P < 0.001$), respectively, but not hepatic cholesterol as compared to FL group. Moreover, this decrease in hepatic TG was obviously greater than that with metformin monotherapy ($P < 0.01$). It increased the hepatic tocopherol-alpha/MDA ratio by 233% compared to FL group ($P < 0.01$), and most importantly, reduced hepatic fat content by 46% ($P < 0.01$). Rosiglitazone reversed the liver weight/body weight ratio approximately close to CL because of the decrease in the liver weight rather than increase in total body weight (Table 2). A

Table 2 Plasma cytokines, lipids, and biochemical parameters at the end of the study period (wk 15)

Parameters	MCDD FL	MCDD Rosiglitazone	MCDD Metformin	MCDD M+R	MCDD Ezetimibe	MCDD Valsartan	MCDD R+M+V	MCDD R+M+V+E	P
% changes W/BW	+68	+12	+28	+20	+28	+16	+20	+28	< 0.001
ALT (nkat/L)	800 ± 33	684 ± 16.5	650 ± 33	667 ± 16.5	684 ± 49.5	734 ± 49.5	684 ± 33	717 ± 33	NS
ALB (g/L)	33 ± 7	34 ± 8	33 ± 16	34 ± 15	33 ± 6	33 ± 8	38 ± 5	35 ± 2	NS
Cholesterol (mmol/L)	123 ± 13	148 ± 13	145 ± 17	106 ± 15	100 ± 7	121 ± 16	139 ± 10	97 ± 17	< 0.001
Triglyceride (mmol/L)	0.8 ± 0.1	0.33 ± 0.08	0.5 ± 0.1	0.4 ± 0.1	0.35 ± 0.07	0.4 ± 0.04	0.5 ± 0.06	0.43 ± 0.8	< 0.03
TNF-α (ng/mL)	17.8 ± 10.0	19 ± 15	10 ± 6	30 ± 26	12 ± 7	9 ± 7	10 ± 6	13 ± 12	< 0.01
HOMA-IR	18 ± 2	11 ± 1	12 ± 1	9 ± 0.5	13 ± 1	16 ± 2	9 ± 0.5	8 ± 1	< 0.001
% change	+157	-39	-33	-50	-28	-11	-50	-55	

Table 3 Hepatic lipid composition, MDA, and alpha-tocopherol levels (mean ± SD, n = 6)

Parameters	MCDD FL	MCDD Rosiglitazone	MCDD Metformin	MCDD M+R	MCDD Ezetimibe	MCDD Valsartan	MCDD R+M+V	MCDD R+M+V+E	P
Triglyceride (mol/g liver)	21.8 ± 1.0	9.9 ± 1.4	13.1 ± 1.4	7.9 ± 1.9	10.1 ± 1.5	9.9 ± 1.7	6.9 ± 1.2	7.9 ± 2.1	< 0.0001
% change	+1263	-54	-40	-64	-53	-54	-68	-64	
Cholesterol (mol/g liver)	1.6 ± 0.2	1.6 ± 0.2	1.6 ± 0.1	1.2 ± 0.2	1.2 ± 0.4	1.9 ± 0.3	1.3 ± 0.3	1.1 ± 0.4	
% change	+245	0	0	-27	-25	+19	-19	-31	< 0.003
MDA (mol/g liver)	2.01 ± 0.04	1.2 ± 0.1	0.7 ± 0.1	0.9 ± 0.01	1.1 ± 0.1	1.2 ± 0.2	0.8 ± 0.1	0.6 ± 0.1	< 0.001
% change	+235	-40	-65	-55	-45	-40	-60	-70	
Tocopherol- α (mg/g liver)	0.07 ± 0.03	0.13 ± 0.04	0.2 ± 0.1	0.2 ± 0.04	0.34 ± 0.09	0.14 ± 0.02	0.19 ± 0.04	0.38 ± 0.8	< 0.001
% change	-77	+86	+186	+186	+386	+100	+171	+443	
Tocopherol-α /MDA ratio	0.003	0.01	0.028	0.02	0.03	0.011	0.023	0.06	< 0.001
% change	-94	+233	+833	+566	+900	+266	+666	+1900	

significant inverse correlation ($r = -0.6$) was found between hepatic alpha-tocopherol and hepatic MDA concentrations in the rosiglitazone group. Rosiglitazone did not significantly reduce the TNF-α levels as compared to FL group.

Metformin markedly reduced insulin resistance (-33%, $P < 0.03$), decreased hepatic TG (-40%, $P < 0.01$) and most effectively improved the tocopherol-alpha/MDA ratio (+833%, $P < 0.03$) as compared to FL group. However, it only mildly decreased the hepatic fat content (-14%, $P < 0.05$). Metformin decreased serum TNF-α levels by 44% ($P < 0.01$) when compared to FL group, and reduced hepatic TG to a lesser extent than rosiglitazone and had no effect on hepatic cholesterol content.

The combination of metformin and rosiglitazone was beneficial in many ways: it reduced insulin resistance by -50% ($P < 0.03$) and decreased hepatic and plasma TG by -64% ($P < 0.02$) and by -50% ($P < 0.03$) respectively; decreased hepatic cholesterol by -27% as compared to FL group ($P < 0.01$) and a significant increase in tocopherol-alpha/MDA ratio (566%, $P < 0.01$) as well as a 32% reduction in hepatic fat ($P < 0.03$) were observed. Surprisingly, this reduction of liver fat content was less important than rosiglitazone monotherapy (32% *vs* 46%, $P < 0.01$) but greater than metformin monotherapy (32% *vs* 14%, $P < 0.001$). And also, the combination of ISAs significantly increased TNF-α levels when compared to FL alone ($P < 0.02$, Tables 2 and 3).

Effect of ezetimibe

Ezetimibe, a cholesterol absorption inhibitor, reversed the liver weight/body weight ratio and improved insulin resistance by -28% ($P < 0.03$), improved oxidant-antioxidant status, reduced TNF-α levels and mildly decreased the hepatic fat content (-14%). Ezetimibe also decreased hepatic and plasma triglycerides by 53% ($P < 0.01$) and 56% ($P < 0.03$), respectively when compared to FL rats. In addition, a 25% decrease ($P < 0.03$) in hepatic cholesterol and a 39% decrease ($P < 0.02$) in plasma cholesterol were observed when compared to CL group, suggesting an apparent effect on cholesterol absorption. Of all aforementioned drugs, ezetimibe monotherapy exerted the most powerful antioxidant effect, whereby it caused the highest increase in the alpha-tocopherol/MDA ratio (900%, $P < 0.001$) followed by metformin monotherapy (+ 833%). Ezetimibe also decreased TNF-α level by 32% ($P < 0.01$) and hepatic fat content by 14% ($P < 0.01$) (Tables 2 and 3). This mild reduction in hepatic fat content might be partially related to a significant effect on cholesterol absorption.

Effect of valsartan

Although valsartan had no significant effect on insulin resistance, it decreased hepatic and plasma triglycerides by -54% ($P < 0.02$) and -50% ($P < 0.01$), respectively and improved the tocopherol-alpha/MDA ratio by +266% ($P < 0.02$). However, it had no beneficial effect on hepatic

Table 4 Liver histological injury: Grading and staging (mean \pm SD, $n = 6$)

Liver histology	Chow	MCDD Only	MCDD Rosiglitazone	MCDD Metformin	MCDD M+R	MCDD Ezetimibe	MCDD Valsartan	MCDD R+M+V	MCDD R+M+V+E	P
Steatosis %	0	91 \pm 5	45 \pm 5	74 \pm 11	59 \pm 11	77 \pm 8	63% \pm 8	51 \pm 4	37 \pm 15	< 0.001
Lobular inflammation	0.5 \pm 0.1	0.7 \pm 0.1	0.7 \pm 0.1	0.6 \pm 0.1	0.6 \pm 0.1	0.7 \pm 0.1	0.5 \pm 0.1	0.5 \pm 0.1	0.6	NS
Portal inflammation	None	None	None	None	None	None	None	None	None	NS
Fibrosis (> F1)	None	None	None	None	None	None	None	None	None	NS
# Rats w/MB	0	2	1	2	2	1	1	1	1	NS
Ballooning	0	2	1	1	1	0	1	1	2	NS
Pericellular fibrosis	0	1	0	1	2	1	0	2	1	NS

MB: Mallory body; NS: Non-significant.

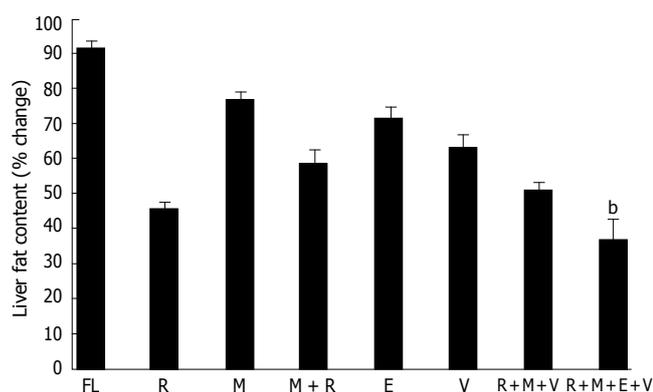


Figure 2 Effect of 6-wk rosiglitazone, metformin, ezetimibe, valsartan, or the combination of all the above drugs on percentage (%) of liver fat content in rats fed with MCD diet.

cholesterol (+ 19%). Finally, it significantly decreased hepatic fat content (-28%) when compared to FL fed with MCCD ($P < 0.01$). Valsartan did not add much to the effect of metformin and rosiglitazone combination therapy regarding fat content but had significant effect on TNF- α levels (-49%, $P < 0.01$). The dosage used in this study was low and demonstrated no significant effect on blood pressure (Tables 2 and 3).

Effect of combined drug therapy

Combined drug therapy was most effective in improving the insulin resistance index (-55%, $P < 0.001$) followed by the combination of insulin-sensitizing agents alone (-50%, $P < 0.02$). The combined administration of drugs caused similar decrease in hepatic and plasma triglycerides as the combination of ISA alone (Tables 2 and 3). Furthermore, the combination therapy decreased hepatic and plasma cholesterol by 31% ($P < 0.02$) and 21% ($P < 0.03$), respectively when compared to FL. They demonstrated a powerful antioxidant activity by decreasing hepatic MDA levels and increasing tocopherol-alpha/MDA ratio by 1 900% ($P < 0.01$ and $P < 0.02$, respectively). Finally and most importantly, the combination of all the above drugs improved hepatic steatosis to a greater extent than ISA alone (54% *vs* 32%, $P < 0.001$) and greater than rosiglitazone monotherapy (54% *vs* 46%, $P < 0.01$). Combined therapy also decreased the serum TNF- α level

by -27% (Tables 2 and 3, Figure 2). The combination of all drugs had a greater effect on hepatic tocopherol-alpha, hepatic MDA, and hepatic cholesterol than the combination of three drugs (R+M+V) (Table 3). The effect of various drugs on hepatocellular injury is shown in Table 4.

DISCUSSION

There are currently no approved therapeutic regimes for treatment of NAFLD. Given the strong associations of NAFLD with features of the metabolic syndrome, including insulin resistance, hyperlipidemia and hypertension, it is reasonable to suppose that agents ameliorating these conditions might also attenuate the development of NAFLD, and considering also that NAFLD is likely to be multifactorial in etiology, it is also sound to predict that combination treatment may be more effective than monotherapy. The present study clearly indicates that combination of insulin-sensitizing agent, cholesterol absorption inhibitor and angiotensin antagonist have greater effect on liver fat content and on lipid peroxidation than monotherapy in the MCDD rat model of NAFLD. Hepatic steatosis and hepatic TG accumulation lead to hepatic insulin resistance by stimulating gluconeogenesis and by activation of protein kinase PKC and JNK-1, which may interfere with tyrosine phosphorylation of IRS-1 and IRS-2 and impair the ability of insulin to activate glycogen synthase and diminish the ability of the liver to store glucose as glycogen^[30]. Rosiglitazone appears to improve hepatic steatosis to a greater extent than metformin and the same effect was observed when examining hepatic triglyceride but not hepatic cholesterol content. The present study is in keeping with previous data showing a similar decrease in liver fat using either pioglitazone^[31] or rosiglitazone^[32,33]. In a human study, rosiglitazone decreased liver fat content by 22% as assessed by magnetic resonance spectroscopy^[34]. Several mechanisms may underlie the ability of rosiglitazone to reduce liver fat content. First, it has been suggested that rosiglitazone doubles fatty acid uptake into subcutaneous adipose tissue and decreases hepatic fatty acid uptake into liver by 40% and in muscle by 30%, i.e., "the lipid steal mechanism"^[35]. Second, rosiglitazone activates PPAR-gamma which decreases free

fatty acid availability for hepatic lipogenesis and reduces the release of TNF- α cytokine by adipocytes^[16,36]. Finally, rosiglitazone has also been shown to increase insulin sensitivity by improving downstream insulin signaling by increasing insulin stimulation of IRS-1 tyrosine phosphorylation and p85 association with IRS-1^[37]. Consistent with the reduction of free fatty acids flux to the liver is the decrease in hepatic TG concentration^[35] and the improvement in insulin sensitivity. Rosiglitazone may also increase adiponectin expression which in turn increases hepatic insulin sensitivity, activates fatty acid oxidation and inhibits phosphoenolpyruvate carboxykinase expression^[38]. Unfortunately, concentrations of adiponectin and free fatty acid were not measured in this study.

We found metformin sensitizes the liver but has little effect on hepatic fat content. The ability of metformin to increase hepatic insulin sensitivity has been documented in a previous study^[39], but liver fat content was not measured. In isolated hepatocytes and metformin-treated rats, lipogenesis was inhibited because of adenosine 5' monophosphate-activated protein kinase (AMPK)-induced inactivation of acetyl-CoA carboxylase and suppression of lipogenic enzyme and transcription factor expression^[40]. Additionally, metformin has been shown to inhibit oxidative phosphorylation and lower cellular ATP levels at high doses^[41]. The dose used in the present study (200 mg/kg) was smaller than that used in the study by Zhou *et al*^[40] (286 mg/kg). In ob/ob mice with FL, metformin was found to reverse hepatomegaly and steatosis^[41]. The therapeutic mechanism of metformin likely involves hepatic expression of TNF- α and TNF- α inducible factors that promote hepatic lipid accumulation and ATP depletion^[42]. In the current study, plasma TNF- α decreased with metformin when compared to untreated FL. It remains to be determined whether metformin also increases adiponectin levels or impacts on free fatty acid uptake and distribution. Although rosiglitazone and metformin activate different signaling pathways, there was no additive effect of the combination of both drugs on liver fat content compared to rosiglitazone monotherapy. In contrast, there was an additive effect of the combination of ISA on hepatic triglyceride, hepatic cholesterol content, and on anti-oxidant/oxidant ratio (Table 3); the reason for this is unclear. It could be related to up-regulation of TNF- α expression which was observed in the R+M group. There was no significant increase in body weight by combined rosiglitazone and metformin therapy. This effect may have been counterbalanced by metformin treatment.

Ezetimibe inhibits the transport of dietary and biliary cholesterol across the intestinal wall^[43]. It was chosen rather than a statin because statins are not very effective in the treatment of NAFLD (atorvastatin) and because ezetimibe is safe and may help in reducing the cholesterol absorption from food intake. The other rationale for using ezetimibe was the high corn oil component (100 g/kg) in the MCD diet. Ezetimibe decreased hepatic fat content with an apparent mild decrease in cholesterol absorption, suggesting that its antioxidant and anti-inflammatory effects are via other mechanisms for reducing liver fat. The dose used in our experiment is considered high dose because the effective dose at which 50% inhibition is

observed (ED₅₀) for rat is 0.0016 mg/kg. This dose was chosen in order to capture any effect ezetimibe might have on the hydrolysis of cholesterol ester^[43]. Usually, ezetimibe does not affect the acute absorption of TG^[44]. In our study, ezetimibe, however, reduced hepatic and plasma TG, which is in accordance with the companion study in which ezetimibe decreased the plasma concentration of TGs^[45].

Treatment with valsartan had modest antioxidant activity as compared to ezetimibe. The molecular mechanism was not investigated in this study, but Siuchi *et al*^[17] have postulated that valsartan treatment exaggerates the insulin-induced phosphorylation of IRS-1, the association of IRS-1 with the p85 regulatory subunit of PI3-K activity and translocation of GLUT 4 to the plasma membrane. Whether valsartan has a selective additional PPAR- γ modulating activity as Telmisartan remains to be determined^[46]. The therapeutic efficacy of another angiotensin 11 receptor antagonist in patients with NASH has recently been documented^[47]. The antifibrotic effect of valsartan was not seen in our rats because rats were fed with the MCCD for a short time (9 wk) and did not produce enough fibrosis.

Abrogation of oxidative stress improves whole body insulin resistance^[48]. In this respect, our study showed that alpha-tocopherol/MDA ratio improved mostly with the combination of all drugs, followed by ezetimibe and by metformin (+1900, +900%, +833%, respectively). Hypertriglyceridemia and increased hepatic TG were prominent features in rats fed with MCDD^[19,20]. Nevertheless, reduction of TG did not improve liver fat content in all study groups. For example, metformin reduced hepatic TGs and hepatic steatosis by 40% and 17%, respectively. This implies that another mechanism of hepatic fat reduction is implicated in addition to lowering hepatic triglycerides. The mean levels of plasma TNF- α were relatively low in all treatment groups because rats were examined in early phase of the disease progression and with mild overt NASH with inflammation, ballooning and pericellular fibrosis. The reason for increased TNF- α in rats fed with MCDD diet with rosiglitazone plus metformin is unclear, but appears to be unrelated to overweight or to lipid peroxidation in any case. Whether it is related to an early activation of NF-kappa B or to a potentiation of IL-6 and IL-8 expression by rosiglitazone plus metformin treatment remains unknown^[49].

Although the MCDD model of fatty liver is the most commonly used in experimental studies, one limitation of our study is that MCDD model is not the ideal model for studying insulin-sensitizing drugs and an extensive number of additional experiments would be required to perform in order to derive definitive data in insulin-resistant models^[50]. Another concern is that rats fed with MCDD for 9 wk did not develop the same phenotype as previous reports of MCDD-induced NASH in mice (as short as 10 d of MCDD). The phenotype described here largely consisted of marked steatosis with mild inflammation and mild fibrosis^[51].

In conclusion, the current study shows for the first time that combination therapy of rosiglitazone and metformin with ezetimibe and valsartan in parallel has a greater effect on the extent of fatty infiltration and on lipid

peroxidation compared to insulin-sensitizing agents only and to monotherapy with either drug. Improving insulin resistance together with decreasing TNF- α and reduction of oxidative stress remains the best targeted treatment to date for NAFLD.

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Prognostic significance of microsatellite alterations at 1p36 in cholangiocarcinoma

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nerve invasion ($P = 0.029$). Moreover, patients who demonstrated MSI at D1S228 showed a poor prognosis ($P = 0.0026$).

CONCLUSION: Allelic loss plays a major role in microsatellite alterations at chromosome 1p36, which may contribute to carcinogenesis and pathogenesis of liver fluke related CCA and these alterations can be used as molecular prognostic indicators for CCA patients.

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Key words: Cholangiocarcinoma; Liver fluke; Chromosome 1p36; Loss of heterozygosity; Microsatellite instability

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Abstract

AIM: To investigate loss of heterozygosity (LOH) and microsatellite instability (MSI) on the chromosomal region 1p36-pter in cholangiocarcinoma (CCA) patients and determine the association between microsatellite alterations and clinicopathological parameters.

METHODS: Ten polymorphic microsatellite markers were determined for LOH and MSI using GS-3000 gel scan fragment autoanalyzer.

RESULTS: Sixty-eight out of 90 cases (75.6%) showed LOH in one or more loci. LOH was found most frequently at D1S199 (40.0%), D1S507 (34.6%), D1S2845 (30.5%), and D1S2734 (30.1%). MSI was found in 34 of 90 cases (37.8%) at one or more loci. Fine mapping at 1p36 showed two distinctive regions of common loss, which were D1S2845 and the 25.5-cM region between D1S507 and D1S2734, indicating the existence of putative tumor suppressor genes that is likely to play important roles in the development of CCA. Patients with LOH at D1S234 showed less lymphatic invasion ($P = 0.017$), whereas patients with LOH at D1S2676 exhibited more lymphatic invasion than those without ($P = 0.031$). LOH at D1S2845 showed a significant correlation with

INTRODUCTION

Cholangiocarcinoma (CCA) is a malignant tumor arising from bile duct epithelium and commonly found in the northeastern region of Thailand with the highest incidence in Khon Kaen^[1,2]. In Thailand, chronic infection with the liver fluke, *Opisthorchis viverrini* (OV), is the major risk factor for the development of CCA^[3]. Prolonged inflammation induced by parasitic infection causes continuous production of free radicals that not only combat the infection, but also act as carcinogens causing mutations and chromosomal breakage and aberrations within the body^[4,5]. Patients with CCA are often diagnosed clinically and cholangiographically but mostly are in advanced stage and difficult to cure successfully. The prognosis of CCA is extremely poor^[6]. Moreover, the molecular events involved in the development of CCA are not well understood.

Microsatellites are short tandem repeat sequences of unknown function scattered throughout the human genome. Loss of heterozygosity (LOH) and microsatellite instability (MSI) are the phenotypes of genetic instability caused by the abnormalities of tumor suppressor and DNA mismatch repair (MMR) genes, respectively. MSI

Table 1 Sequences of microsatellite markers located on the chromosomal region 1p36-pter used for the LOH and MSI analysis

Marker	Sequences (5'-3')
D1S468	F: AAT TAA CCG TTT TGG TCC T R: GCG ACA CAC ACT TCC C
D1S2845	F: CCA AAG GGT GCT TCT C R: GTG GCA TTC CAA CCT C
D1S450	F: GCT CCA ATG TCC AAG GG R: GGG TAC TCA GAT GGC TGG T
D1S228	F: AAC TGC AAC ATT GAA ATG GC R: GGG ACC ATA GTT CTT GGT GA
D1S507	F: AGG GGA TCT TGG CAC TTG G R: CTC TAG GGT TTC TGG AAA ATG CTG
D1S199	F: GGT GAC AGA GTG AGA CCC TG R: CAA AGA CCA TGT GCT CCG TA
D1S2734	F: GGT TCA AGG GAT TCT CCT G R: TGG CAC TCA GAC CTC AA
D1S234	F: GCC CAG GAG GTT GAG G R: AAG GCA GGC TTG AAT TAC AG
D1S2781	F: CTC TCA CAG ACA CAC GCA R: GTT CAA TGG GGG ATT CAG
D1S2676	F: TCT GTC AGA ACA AAC GTG TC R: GAG TTG CCA TAC TTT GCT GTA G

is associated with slippage of DNA polymerase during DNA synthesis resulting in changing units of repetitive sequences. The instability of microsatellites is detected by the difference of the lengths of the repeat sequences between tumor and normal DNA from the same patient. As allelic loss at a certain region of chromosome is thought to indicate the presence of a tumor suppressor gene, LOH analysis is presently the most common method used to identify potential locations of these genes.

Frequent allelic losses on specific chromosomal regions have been reported in CCA^[7-11]. On the other hand, only a few studies on microsatellite instability have been performed in this cancer^[12-15]. Our data on comparative genomic hybridization (CGH) in CCA showed the most frequent decrease in DNA copy number at 1p36-pter (34%). Moreover, the chromosomal region 1p36 appears to harbor critical tumor suppressor genes important to tumorigenesis and progression in many human cancers^[16-20]. Therefore, this study aimed to investigate the incidence of LOH and MSI at chromosome 1p36-pter in liver fluke related CCA using 10 polymorphic microsatellite markers. We also determined the correlation between microsatellite alterations and clinicopathological parameters.

MATERIALS AND METHODS

Patients and DNA preparation

Blood and liver resection samples were obtained from 90 patients with intrahepatic cholangiocarcinoma who underwent surgery at Srinagarind Hospital, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand. Informed consent was obtained from each patient according to the guidelines of the Ethical Committee of Khon Kaen University. DNA was extracted from microdissected tissues using DNA isolation kit according to manufacturer's instructions (Puregene, Gentra systems,

USA). Matched peripheral blood leucocytes were prepared for DNA by the method described previously^[13].

Microsatellite markers

Ten microsatellite markers specific for the chromosomal region 1p36-pter were used. The forward primer of each marker was labeled at 5' end with 4,7,2',4',5',7'-hexachloro-6-carboxyfluorescein (HEX). Primer sequences were obtained from the Human Genome Database as shown in Table 1. D1S468 is located at telomeric end while D1S2676 is located toward centromeric end.

PCR amplification

PCR was performed in a 25 μ L reaction containing 50 ng of DNA, 100 μ mol/L of each deoxynucleoside triphosphate (dNTP), 5 pmol of each primer, 3 mmol/L MgCl₂, 50 mmol/L KCl, 10 mmol/L Tris HCl pH 8.3, and 1.5 units of Taq DNA polymerase. Additives such as 0.4 mL/L Triton X-100 or 0.4 mL/L Tween 20 were added in the amplification of tissue DNA. The PCR conditions were denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 30 s (or at 56°C for D1S468 and D1S2845, at 59°C for D1S199, and at 54°C for D1S2734), extension at 72°C for 30 s, and final extension at 72°C for 10 min.

Analysis of LOH and MSI

PCR products were denatured by 950 mL/L formamide and electrophoresed on 6% or 8% polyacrylamide gels containing 7 mol/L urea using GS-3000 gel scan fragment autoanalyzer (Corbett Research, Australia). MSI was defined as changes of size bands observed in tumor DNA but not visible in the corresponding normal DNA. For informative cases, allelic loss (LOH) was assessed if the signal of one allele was at least 50% reduced in the tumor DNA as compared with the corresponding normal allele. Our previous study^[13] showed the same pattern between non-tumorous tissue and leucocyte DNA, therefore in this study only leucocyte DNA was used as the corresponding normal DNA. Each assay was performed twice in order to ensure experimental reproducibility.

Statistical analysis

LOH and MSI frequencies were correlated with several clinical parameters using χ^2 test. Survival curves were calculated using Kaplan-Meier and log rank test. The result was considered statistically significant when $P < 0.05$.

RESULTS

LOH and MSI analysis

The percentage of LOH was analyzed based on informative cases while that of MSI could be analyzed within 90 cases. Representative examples of LOH and MSI at various loci are shown in Figure 1. LOH at one or more loci was observed in 68 out of 90 cases (75.6%). If a percentage of LOH at a locus was more than 30%, we determined that LOH frequency of the locus was significantly high. The percentage of LOH at each microsatellite marker varied from 17.3% to 40.0%. Among 10 loci, we observed

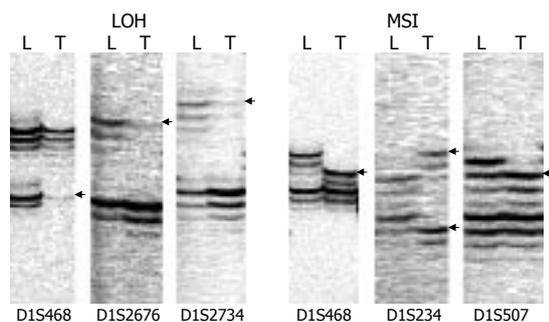


Figure 1 Representative examples of LOH and MSI at various loci of chromosome 1p36 (L, leucocyte DNA; T, tumor DNA). Arrows indicate the abnormalities

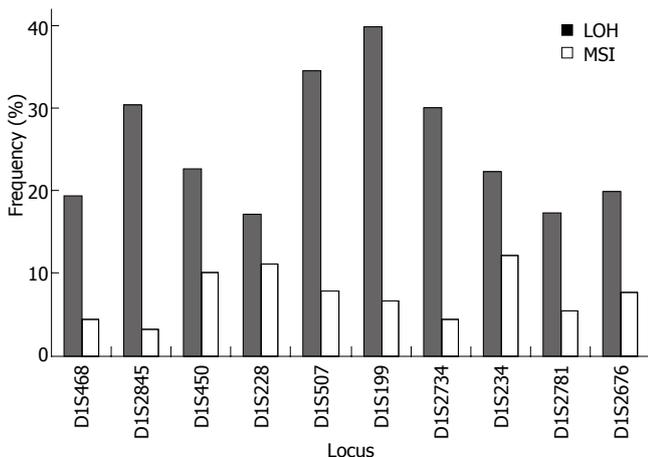


Figure 2 LOH and MSI frequencies at each locus of chromosome 1p36-pter.

4 loci with significantly high LOH frequency, which were D1S199 (40.0%), D1S507 (34.6%), D1S2845 (30.5%), and D1S2734 (30.1%). MSI was found in 34 (37.8%) of 90 cases at one or more loci. Among 10 loci, 3 loci showed percentage of MSI more than 10%, which were D1S234 (12.2%), D1S228 (11.2%), and D1S450 (10.1%). The percentages of LOH and MSI at each locus are shown in Figure 2. Interestingly, the percentage of MSI at each locus was much less than that of LOH, suggesting that LOH is a preferable pathway to MSI for the alterations of microsatellite markers on the chromosome 1p36 in liver fluke associated CCA.

Fine mapping of chromosomal region 1p36-pter

Fine mapping of the chromosomal region 1p36-pter in 90 CCA cases is shown in Figure 3. Four distinctive loci with high LOH frequencies were denoted giving rise to two common loss regions; D1S2845 and the 25.5-cM region between D1S507 and D1S2734.

Correlation between microsatellite alterations and clinicopathological data

The associations between LOH and MSI at each locus and clinicopathological parameters of CCA patients were analyzed. Patients with LOH at D1S2845 showed significantly more nerve invasion than those without ($P = 0.029$). Samples with moderately differentiated adenocarcinoma rarely found LOH at D1S234 ($P = 0.035$).

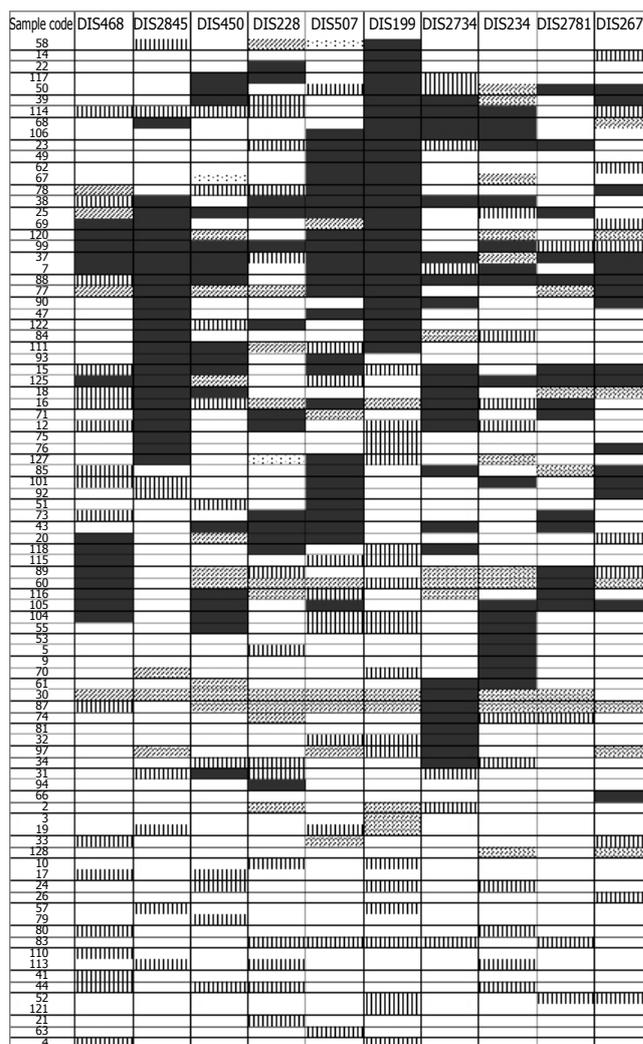


Figure 3 Fine mapping of the chromosome 1p36-pter in 90 CCA. The common loss regions were located at D1S2845 and the 25.5-cM region between D1S507 and D1S2734. — Normal; ■ LOH; ▨ MSI; ▤ Non informative; ▧ PCR failed

Patients who had LOH at D1S234 exhibited significantly less lymphatic invasion ($P = 0.017$), whereas patients with LOH at D1S2676 showed significantly more lymphatic invasion ($P = 0.031$). Clinical features of patients with and without LOH at loci D1S2845, D1S234, and D1S2676 are summarized in Table 2. A survival curve by the Kaplan-Meier method showed a poor prognosis for patients who demonstrated MSI at D1S228 (Figure 4). The log-rank test confirmed a significant association between poor prognosis and MSI at D1S228 ($P = 0.0026$).

DISCUSSION

Microsatellite polymorphisms provide a large number of highly informative loci, which may allow simultaneous, rapid screening of LOH and MSI using very small amounts of DNA samples. Our finding of LOH at one or more loci was observed in 68 (75.6%) out of 90 cases. This finding is different from that of Momoi *et al*¹¹. They determined fine mapping of chromosome 1p36 using 13 markers in 22 CCA patients and showed LOH at one or more loci in 13 (59.1%) cases. The differences

Table 2 Clinical features of patients with and without LOH at loci D1S2845, D1S234, and D1S2676

Clinical parameters	D1S2845				D1S234				D1S2676			
	n ¹	Without LOH n (%)	With LOH n (%)	P	n ¹	Without LOH n (%)	With LOH n (%)	P	n ¹	Without LOH n (%)	With LOH n (%)	P
Number of patients	82	57 (69.5)	25 (30.5)		80	62 (77.5)	18 (22.5)		80	64 (80.0)	16 (20.0)	
Age range (yr)												
32-53	36	23 (63.9)	13 (36.1)	NS	34	26 (76.5)	8 (23.5)	NS	37	31 (83.8)	6 (16.2)	NS
54-75	46	34 (73.9)	12 (26.1)		46	36 (78.3)	10 (21.7)		43	33 (76.7)	10 (23.3)	
Sex												
Male	55	38 (69.1)	17 (30.9)	NS	54	42 (77.8)	12 (22.2)	NS	54	41 (75.9)	13 (24.1)	NS
Female	27	19 (70.4)	8 (29.6)		26	20 (76.9)	6 (23.1)		26	23 (88.5)	3 (11.5)	
Histological type												
Papillary	19	12 (63.2)	7 (36.8)	NS	17	10 (58.8)	7 (41.2)	0.035	20	17 (85.0)	3 (15.0)	NS
Well diff	22	15 (68.2)	7 (31.8)		23	20 (87.0)	3 (13.0)		22	17 (77.3)	5 (22.7)	
Moderately diff	11	7 (63.6)	4 (36.4)		11	10 (90.9)	1 (9.1)		12	11 (91.7)	1 (8.3)	
Poorly diff	18	14 (77.8)	4 (22.2)		18	16 (88.9)	2 (11.1)		15	11 (73.3)	4 (26.7)	
Rare phenotype ²	12	9 (75.0)	3 (25.0)		11	6 (54.5)	5 (45.5)		11	8 (72.7)	3 (27.3)	
Blood vessel invasion												
Invasion	52	40 (76.9)	12 (23.1)	NS	50	39 (78.0)	11 (22.0)	NS	50	40 (80.0)	10 (20.0)	NS
Non-invasion	30	17 (56.7)	13 (43.3)		30	23 (76.7)	7 (23.3)		30	24 (80.0)	6 (20.0)	
Nerve invasion												
Invasion	36	20 (55.6)	16 (44.4)	0.029	36	30 (83.3)	6 (16.7)	NS	37	27 (73.0)	10 (27.0)	NS
Non-invasion	46	37 (80.4)	9 (19.6)		44	32 (72.7)	12 (27.3)		43	37 (86.0)	6 (14.0)	
Lymphatic invasion												
Invasion	58	38 (65.5)	20 (34.5)	NS	56	48 (85.7)	8 (14.3)	0.017	57	42 (73.7)	15 (26.3)	0.031
Non-invasion	24	19 (79.2)	5 (20.8)		24	14 (58.3)	10 (41.7)		23	22 (95.7)	1 (4.3)	
Survival mean (wk)	82	57.53	34.97	NS	80	59.72	39.23	NS	80	59.78	28.52	NS

¹Number of informative cases; ²Squamous and adenosquamous carcinoma.

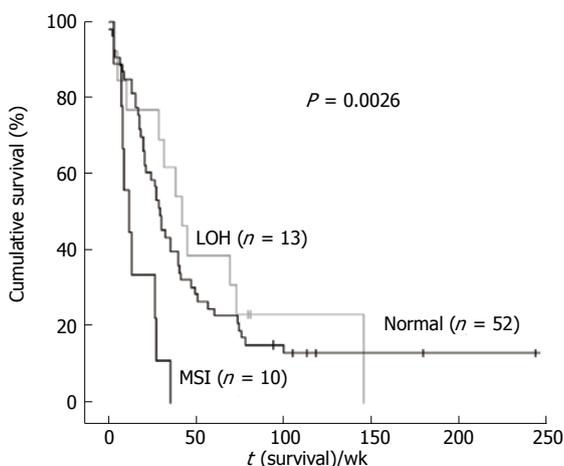


Figure 4 The Kaplan-Meier survival curves showed a poor prognosis in patients who demonstrated MSI at D1S228. Non informative cases were excluded.

in percentage of LOH from Momoi *et al* and our studies suggest the differences in type and number of samples and type and number of markers used. The type of samples studied by Momoi *et al* was frozen tissues which may have dilution effects from normal tissues, whereas the samples in our study were microdissected tissues among which only tumor cells were selected. Our study used a large number of samples (90 cases). The number of markers used in our study was 10 with 5 markers similar to those studied by Momoi *et al*, suggesting that the locations of different markers may lead to different percentages. However, by comparison of 5 similar markers used in Momoi *et al* and our studies, the LOH frequencies at D1S450 (5%),

D1S507 (18.7%), and D1S199 (22.2%) detected by Momoi *et al* were lower than those of our study, whereas the LOH frequencies at TP73 (54.5%) and D1S228 (29.4%) were higher than those of our study. This suggests differences in tumor etiology. In our study, all tumors were obtained from patients who were residents of northeastern region of Thailand where liver fluke infection remains highly endemic. Genetic alterations in CCA caused by liver fluke infection may be different from those caused by others such as hepatolithiasis and primary sclerosing cholangitis. The different percentages also suggested the specificity of genetic alterations in each tumor type.

The *p73* gene is mapped to 1p36 at D1S468 and *p73* protein is structurally similar to the *p53* protein within its DNA-binding, transactivating, and oligomerization domains but C-terminal extension contains Sterile Alpha Motif (SAM) domain^[21,22]. The *p73* protein also shares some functional characteristics with *p53*, including the ability to promote apoptosis when overexpressed *in vitro* and up-regulate *p53* responsive genes involved in cell-cycle control such as *p21*^[23]. In neuroblastomas, the incidence of *p73* LOH was found to be significantly higher in advanced tumors than in earlier stage^[24]. In breast carcinomas, LOH in the *p73* region could be pathogenically related to breast cancer and possibly to a poor tumor prognosis^[25]. A relatively high frequency of LOH at D1S468 (19.4%) in our study suggests that inactivation of *p73* may play a role in CCA development. Fine mapping of chromosome 1p36 showed two regions of common allelic loss. The first region was at D1S2845 and the second region was from D1S507 to D1S2734 loci. A relative large area of deletion involving markers D1S507, D1S199, and

D1S2734 encompasses a 25.5-cM region. Candidate tumor suppressor genes located at D1S2845, D1S507, D1S199, and D1S2734 are DNA fragmentation factor, 40 ku, beta polypeptide (*DFFB*); caspase 9, apoptosis-related cysteine protease (*CASP9*); paired box homeotic gene (*PAX7*); and inhibitor of DNA binding 3 (dominant negative helix-loop-helix protein, *ID3*), respectively.

Statistical analyses were performed to determine whether the frequency of individual microsatellite marker showing alterations was correlated with clinical parameters. We found that MSI at marker D1S228 showed a significant correlation with poor survival ($P = 0.0026$). On the physical map, the retinoblastoma interacting zinc finger gene (*RIZ*) is adjacent to the polymorphic marker D1S228^[17]. In addition, *RIZ* harbors intragenic microsatellites and polyadenosine tracts within its coding region, it is a candidate for an inactivating mutation in MSI mediated carcinogenesis^[26,27]. MMR defect may cause not only MSI at D1S228 but also at the microsatellite coding sequences of *RIZ*, resulting in frame shift mutation of this gene and contributing to poor survival. However, our study showed that LOH at D1S228 did not affect patient survival, suggesting that LOH was not a major pathway of *RIZ* inactivation. An alternative pathway such as mutation and promoter hypermethylation may be a major pathway for *RIZ* inactivation in CCA, on which further study is needed. The high frequency of MSI found in this study (37.8%) suggests the involvement of MMR defect in liver fluke related CCA. However, the frequency of *bMLH1* alteration found in MSI (+) tumors showed no significant difference from that of MSS tumors (data not shown), suggesting that other MMR members may be defective. In contrast, infrequent MSI in CCA was reported by Liengswangwong *et al.*^[13]. This may be due to the differences in types of samples and markers.

D1S2845 located at 1p36.3 showed a significant correlation with nerve invasion ($P = 0.029$). The putative tumor suppressor gene located on this region is *DFFB*, which function is involved in apoptosis^[19,28]. Deletion at this region may lead to tumor progression. Marker D1S234 is located at 1p36.1 in the same region of cell division cycle 42, *Cdc42*. *Cdc42*, a member of the Rho subfamily, is plasma membrane-associated small GTPase, which cycles between an active GTP-bound and an inactive GDP-bound state. In active state, it binds to a variety of effector proteins to regulate cellular responses involved in epithelial cell polarization processes. *Cdc42*, Rac and RhoA influences cell shape and structure by initiating actin cytoskeleton remodeling. RhoA and Rac1 generate actin stress fibers and lamellipodia/membrane ruffles, respectively, whereas *Cdc42* stimulates the formation of microspikes and filopodia^[29]. Loss of D1S234 showed less lymphatic invasion, suggesting that lack of *Cdc42* may cause tumor cells to be less invasive ($P = 0.017$). Our previous study on microsatellite alterations at loci D2S119, D3S1611 and TP53 showed the association with poor prognosis^[13]. The previous and present studies indicate the value of microsatellite alterations as prognostic indicators for liver fluke related CCA. With regard to the significant correlations of microsatellite alterations with clinicopathological parameters such as survival, lymphatic

invasion and nerve invasion in this study, we propose the putative tumor suppressor genes; *RIZ*, *Cdc42*, and *DFFB*, which may play important roles in the development and progression of liver fluke related CCA.

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Proximal gastric response to small intestinal nutrients is abnormal in mechanically ventilated critically ill patients

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responses to small intestinal nutrient stimulation are abnormal.

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Abstract

AIM: To determine the response of the proximal stomach to small intestinal nutrients in critically ill patients.

METHODS: Proximal gastric motility was measured in 13 critically ill patients (49.3 ± 4.7 years) and 12 healthy volunteers (27.7 ± 2.9 years) using a barostat technique. Recordings were performed at baseline, during a 60-min intra-duodenal infusion of Ensure[®] (2 kcal/min), and for 2 h following the infusion. Minimum distending pressure (MDP), intra-bag volume and fundic wave activity were determined.

RESULTS: The MDP was higher in patients (11.7 ± 1.1 vs 7.8 ± 0.7 mmHg; $P < 0.01$). Baseline intra-bag volumes were similar in the 2 groups. In healthy subjects, a 'bimodal' proximal gastric volume response was observed. In patients, the initial increase in proximal gastric volume was small and delayed, but eventually reached a maximal volume similar to that of healthy subjects. In healthy subjects, the proximal gastric volume rapidly returned to baseline level after nutrient infusion (median 18 min). In contrast, the recovery of volume to baseline was delayed in critically ill patients (median 106 min). In 6 patients, the volume had not returned to baseline level 2 hours after nutrient infusion. In patients, fundic volume waves were less frequent ($P < 0.05$) and had lower amplitude ($P < 0.001$), compared to healthy subjects.

CONCLUSION: In critical illness, proximal gastric motor

INTRODUCTION

Gastric intolerance to enteral feeding occurs frequently in critical illness and is a major obstruction to the provision of enteral nutrition^[1,2]. Inadequate nutritional support adversely affects both morbidity and mortality^[3,4]. The most common cause of feed intolerance is delayed gastric emptying^[1,2,5], which has a prevalence of up to 50% in tertiary intensive care units^[6]. Moreover, the mechanisms underlying poor gastric emptying in critical illness are not well understood.

Both proximal and distal gastric motility is considered important in normal gastric emptying of liquids^[7,8]. In healthy subjects, there is a relaxation of the proximal stomach, reduction in antro-duodenal motility and an increase in isolated pyloric pressure waves in response to small intestinal feed-back^[9,10]. This feed-back response is triggered by a caloric load of 2-3 kcal/min^[9,10].

In critically ill patients, a marked reduction in antral motility and a poor coordination of antro-duodenal contractions has been reported during fasting^[2,11]. There are, however, no data on the motor activity of the proximal stomach, during fasting or in response to small intestinal nutrients in these patients. The aim of the current study was to assess proximal gastric motor activity during small intestinal nutrient infusion in critically ill patients. We hypothesized that small intestinal feed-back to the proximal stomach in response to duodenal nutrients would be intensified, and increased proximal gastric relaxation would be observed. To minimize a variation in stimulation

due to erratic gastric emptying in critically ill patients, nutrient was infused directly into the duodenum. This provided a constant nutrient delivery at a physiological level to the small intestinal receptors.

MATERIALS AND METHODS

Subjects

Studies were performed in 13 medical, critically ill patients (11 male, mean age 49.3 ± 4.7 years), who were admitted to a level 3 intensive care unit between January and September 2004. Any patient aged greater than 17 years was eligible to be enrolled into the study if they were sedated, mechanically ventilated, required enteral nutrition and had no history of diabetes mellitus. Exclusion criteria comprised recent major abdominal surgery, any contra-indication to the passage of an enteral tube, usage of opioid analgesia, benzodiazepines or prokinetic therapy within the previous 24 h, previous gastric, oesophageal or intestinal surgery, unstable intra-cranial pressure or cervical spine injury. As part of the standard clinical practice in our intensive care unit, all subjects received a sliding scale insulin infusion to maintain blood glucose concentrations between 6 and 8 mmol/L. The demographic characteristics of the subjects are summarized in Table 1.

Studies were also performed in 12 healthy volunteers (8 male, mean age 27.7 ± 2.9 years), none of whom had a history of systemic or gastrointestinal disease, or was taking any medication. Volunteers were instructed to refrain from smoking for 24 h prior to the study.

Written informed consent was obtained from healthy subjects and the next of kin of the critically ill patients prior to enrolment into the study. The protocol was approved by the Human Research Ethics Committee of the Royal Adelaide Hospital.

Measurements

Proximal gastric motility: Proximal gastric motility was measured using an electronic gastric barostat (Distender Series II; G&J Electronics, Ontario, Canada)^[12,13]. A thin flaccid-walled bag with a maximum capacity of 1000 mL was attached to the distal end of the assembly, which was connected to the system via pressure and volume ports. Changes in proximal gastric volume were measured indirectly by changes in the volume of the polyethylene bag.

Data were stored onto a Powermac 7100 computer (Apple Computer, Cupertino, CA), using custom-written data-acquisition software (Labview: National Instruments, Austin, TX). This software was also used to program the barostat to perform distensions in stepwise increments^[14]. Recorded data were imported into a display and analysis program (Acqknowledge, Biopac System, Goleta, CA) for manual analysis. Intra-bag volumes were determined at 2 min intervals and the mean baseline volume was measured over 10 min immediately before the infusion. Changes in intra-bag volume during nutrient infusion were calculated as the difference between the actual bag volume and the mean baseline volume. The serial changes in bag volume during the infusion were plotted and compared. Proximal gastric relaxation was indirectly inferred by an increase in

Table 1 Demographic characteristics of the studied subjects

	Critically ill patients (n = 13)	Healthy subjects (n = 12)
Age (yr)	49.3 ± 4.7 ^b	27.7 ± 2.9
Sex (M:F)	11:2	8:4
BMI (kg/m ²)	29.7 ± 1.7	24.1 ± 1.0
APACHE II score		Not applicable
On admission	24.1 ± 1.3	
Study day	24.7 ± 1.8	
Days in ICU prior to study	5.0 ± 0.2	Not applicable
Diagnosis	Head injury (2), motor vehicle accident (2), cardiac arrest and failure(3), acute pancreatitis (2), subdural haemorrhage, uro-sepsis, chronic obstructed airway disease, asthma	Not applicable

^bP < 0.01 vs healthy subjects.

intra-bag volume^[12].

Assessment of fundic slow volume waves was also performed. These were defined as changes in proximal gastric volume of greater than 30 mL that reverted in less than 2 min to a volume within 50% of the previous level^[15]. The number and volume amplitude of fundic slow waves (per 10 min) were determined during fasting and in response to small intestinal nutrient infusion.

Blood glucose concentration: Marked hyperglycaemia is one of the humoral factors that may play a role in mediating small intestinal feed-back and adversely affects gastric motility^[16]. Blood glucose concentrations were measured using a portable glucometer (Precision Plus, Abbott Laboratories, Bedford, USA)^[17] at timed intervals during the study.

Protocol

Patients and healthy subjects were studied after at least 6-hours fasting and in a 30 degree recumbent position. To standardise the sedative regime in patients, propofol alone was used, and opioids, benzodiazepines or prokinetic agents were not administered for 24 h prior to and during the study. In the healthy subjects, the barostat catheter and infusion tube were swallowed and allowed to pass into the correct position spontaneously, without the assistance of endoscopy. After insertion of the barostat catheter to a depth of 55 cm, the balloon was inflated with 400 mL of air and the catheter was pulled back until resistance was felt^[18]. Duodenal nutrient infusion was achieved by inserting a silicon-rubber catheter (Dentsleeve, Adelaide, Australia) with a central feeding lumen and lead-weighted tip into the duodenum. The correct positioning of the infusion catheter was determined by continuous measurement of the antro-duodenal trans-mucosal potential difference (TMPD) gradient^[18]. Radiological confirmation was not performed.

In patients, placement of both the barostat catheter and post-pyloric feeding tube was performed at the bedside with endoscopic assistance, without additional sedation to that required for ventilation. A 12 French x 114 cm naso-duodenal feeding tube (Flexiflo, Abbott,

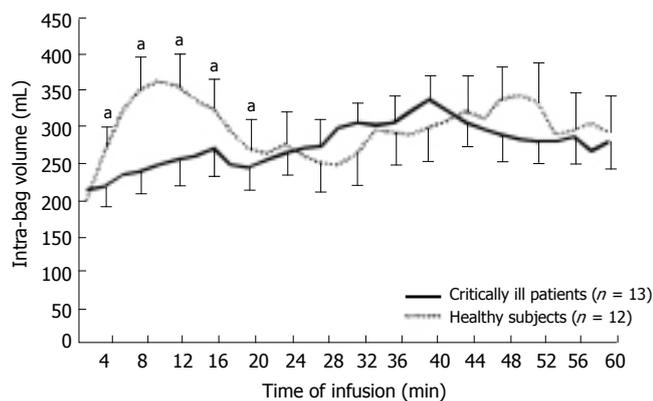


Figure 1 Changes in proximal gastric volume during the 2 kcal/min infusion, in healthy (dotted line, $n = 12$) and critically ill (solid line, $n = 13$) subjects. Data are mean \pm SE. ^a $P < 0.05$ vs healthy subjects.

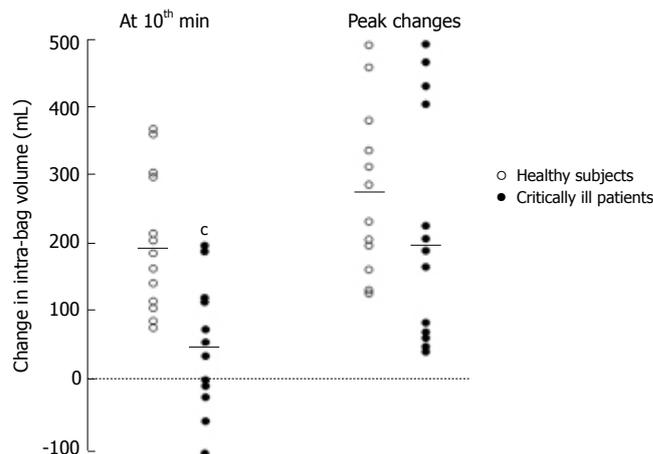


Figure 2 Changes in proximal gastric volume from baseline at the 10th minute and at peak level during the nutrient infusion, in healthy (open circle, $n = 12$) and critically ill (closed circle, $n = 13$) subjects. ^c $P < 0.001$ vs healthy subjects.

Ireland) was inserted into the duodenum over a guide-wire (THSF-35-260, Cook, Australia). The barostat catheter was then guided through the mouth into the stomach by the endoscope. The barostat balloon was inflated with 400 mL of air and gently retracted into the fundus under direct vision. Gastric contents (air and fluid) were completely aspirated prior to withdrawal of the endoscope. Correct placement of the naso-duodenal feeding tube was confirmed at the time of placement by measurement of the TMPD gradient^[18], and subsequently by radiography^[11].

After confirming that the catheters were positioned correctly, air in the barostat balloon was aspirated and the catheter was connected to the barostat. The minimum distending pressure (MDP), defined as the first pressure level that provided an intragastric bag volume of more than 30 mL, was determined^[19]. The baseline pressure for the study was then set at MDP + 2 mmHg^[12]. All studies began with a 15-min baseline recording, during which normal saline was infused into the duodenum at 240 mL/h. Each subject then received a 60-min duodenal infusion of Ensure[®] (Abbott Laboratories, Ohio, USA; nutrient content: 13% protein, 64% carbohydrate, 21% fat; energy density: 1 kcal/mL) at 2 kcal/min. Barostat recordings were performed during the nutrient infusion and continued for 2 h after the infusion was ceased. Blood samples for the measurement of blood glucose concentrations were collected at baseline and at 20-min intervals during the nutrient infusion.

Statistical analyses

Data are presented as mean \pm SE. The differences in demographic characteristics, baseline volume, MDP and fundic volume waves between the healthy subjects and critically ill patients were compared using Student’s unpaired *t*-test. A repeated measures mixed-model analysis of variance (ANOVA) was used to compare the proximal gastric volume and blood glucose responses between the groups, with time and treatment as the factors. Student’s unpaired *t*-test was used to compare the maximum changes in proximal gastric volume between the 2 groups. The time required for the proximal stomach to return to baseline volume following nutrient stimulation was expressed as median and interquartile range (IQR), and differences

between the groups were assessed using the Mann-Whitney test. A $P < 0.05$ was considered statistically significant.

RESULTS

Oral intubation of the assembly was well tolerated by both patients and healthy subjects and no complications occurred in either group. At endoscopy, 2 patients had a small amount of feed residue (< 100 mL) in the stomach and duodenum.

Baseline measurements

The MDP was higher in critically ill patients than in healthy subjects (11.7 ± 1.1 mmHg *vs* 7.8 ± 0.7 mmHg, $P = 0.006$). Baseline proximal gastric volumes were similar between the two groups (patients: 211 ± 48 mL *vs* healthy: 191 ± 24 mL).

Proximal gastric volume response to small intestinal nutrients (Figures 1 and 2)

In the healthy subjects, there was a “bimodal” proximal gastric volume response to small intestinal nutrients, with the first peak occurring within 15 min of the infusion. The proximal gastric volume had reduced by 57 ± 4 % (mean volume reduction = 184 ± 24 mL) at 30 min of the infusion, after which it increased to a second smaller peak at 50 min. In critically ill patients, the increase in proximal gastric volume in response to small intestinal nutrients was initially slower and smaller than in healthy subjects (change in volume at 10th min: 45 ± 26 *vs* 196 ± 29 mL; $P < 0.001$). The proximal gastric volume did not peak until 40 min after the start of the infusion, but eventually reached a similar level to the healthy subjects. The maximal increase in proximal gastric volume did not differ between the two groups (patients: 199 ± 35 mL *vs* healthy: 233 ± 76 mL) (Figures 1 and 2).

Recovery of proximal gastric volume after duodenal nutrient stimulation (Figure 3)

The time course for the proximal stomach to return to baseline volume after nutrient stimulation was assessed

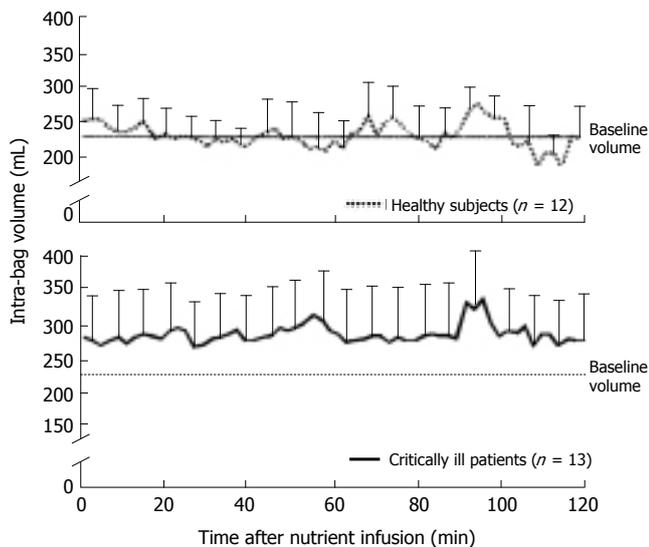


Figure 3 Time course for the proximal gastric volume to return to baseline level after nutrient stimulation, in healthy (dotted line, $n = 12$) and critically ill (solid line $n = 13$) subjects. Data are mean \pm SE.

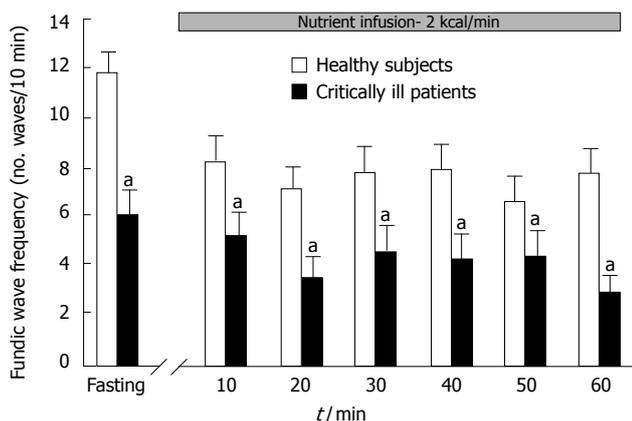


Figure 4 Fundic slow volume waves (per 10 min) during the 2 kcal/min nutrient infusion, in healthy (open bar, $n = 12$) and critically ill (closed bar, $n = 13$) subjects. Data are mean \pm SE. ^a $P < 0.05$ vs healthy subjects.

by analysis of the proximal gastric volume during the 2 hour period following the infusion. In healthy subjects, the proximal gastric volume had returned to baseline level within 30 min after cessation of the nutrient infusion (median = 18 min; IQR: 0-24 min). In patients, the time required for the proximal stomach to return to baseline was significantly longer than in healthy subjects (median = 106 min; IQR: 47-120 min; $P < 0.001$). In only 2 of the 13 patients, the volume had returned to baseline within 30 min of cessation of the infusion. In 6 patients, the proximal gastric volume had not returned to baseline level at 2 h after the nutrient infusion was ceased (Figure 3).

Fundic volume waves during fasting and in response to small intestinal nutrients (Figure 4)

The frequency of fundic volume waves was less in critically ill patients during both fasting and nutrient infusion, compared to healthy subjects ($P < 0.05$) (Figure 4). In addition, the volume amplitude of fundic waves was smaller in patients than in healthy subjects (44 ± 3 mL *vs*

87 ± 8 mL; $P < 0.001$).

Blood glucose concentration

Blood glucose concentrations were higher in critically ill patients at baseline (7.0 ± 0.3 mmol/L *vs* 5.2 ± 0.2 mmol/L; $P < 0.001$) and during nutrient infusion (8.3 ± 0.2 mmol/L *vs* 7.2 ± 0.2 mmol/L; $P < 0.01$), compared to healthy subjects. However, the magnitude of increase in blood glucose concentration during the infusion did not differ between the 2 groups.

DISCUSSION

This is the first study to evaluate the motor responses of the proximal stomach to small intestinal nutrient infusion in critically ill patients. Our data demonstrate that in these patients, (1) proximal gastric relaxation is delayed with no change in the magnitude of the response, (2) fundic wave activity is reduced, and (3) the recovery of proximal gastric volumes to pre-stimulation levels is delayed.

Although delayed gastric emptying is a major clinical problem in critically ill patients, only distal gastric motor activity has been previously examined^[2,11]. However, abnormal proximal gastric motility has been demonstrated in non-critically ill patients with gastroparesis, such as those with diabetes mellitus^[14,20,21], and may play an important role in slowing gastric emptying^[7,8]. Our findings provide a possible mechanism for the delay in liquid gastric emptying frequently found in critical illness. Failure of the relaxed proximal stomach to return to baseline volume after nutrient stimulation in these patients provides a reservoir for retention of gastric residue in the fundus. Such a prolonged pooling of gastric content is likely to contribute to both delayed gastric emptying and gastro-oesophageal reflux^[5,7,8,22]. Fundic volume waves are thought to be involved in the redistribution of proximal gastric content distally for emptying and high fundic wave activity is associated with accelerated gastric emptying of liquids^[23]. It would seem likely that reduced fundic wave activity may contribute to the slowing of gastric emptying.

The mechanisms underlying the changes in proximal gastric motility in critical illness are unknown. The initial delay in proximal gastric relaxation could relate to physical restriction from a combination of positive mechanical ventilation and high intra-abdominal pressure (reflected by a higher MDP) present in critical illness^[24]. However the eventual relaxation to normal values suggests that this is unlikely to be the cause. Impaired gastric accommodation has been described in diabetes mellitus with autonomic neuropathy^[25-27]. Autonomic dysfunction has also been reported in critically ill patients^[28] and could cause both a delay in gastric relaxation and potentially a prolonged recovery in gastric volume via impairment of different components of the autonomic nervous system. In light of recent reports of interactions between inflammatory mediators, neurotransmitters and intrinsic neural pathways^[26,29], it is also possible that increased cytokine production in critical illness may play a role. Sedative drugs have been reported to alter gastric emptying and proximal gastric motility^[30,31]. While propofol inhibits gastric emptying in animals^[30], its effects in humans are

less clear^[31,32]. A small study in humans showed that a combination of propofol and morphine could result in a smaller proximal gastric volume compared to morphine alone, but gastric emptying is similar between the two groups^[31]. The mechanisms underlying the prolonged recovery of the proximal stomach to baseline volume are also unknown. Although hyperglycemia increases proximal gastric relaxation in both healthy and diabetic subjects^[16,17], the elevation of blood glucose levels in our patients was minimal and in the 'physiological hyperglycemia' range, which seems unlikely to significantly contribute to the prolonged relaxation. Similarly, gastric relaxation due to opiate drugs such as morphine^[32] is unlikely to explain our findings, as they were withheld for 24 h prior to the study. The neurotransmitter nitric oxide is important in mediating proximal gastric relaxation^[33]. Nitric oxide synthesis is increased in critically ill patients^[34,35] and could contribute to prolonged proximal gastric relaxation. The discordant findings of impaired relaxation followed by failure to regain normal tone suggest the involvement of multiple mechanisms. Further studies are required to determine the involvement and interaction of potential mechanisms.

The bimodal pattern of proximal gastric relaxation in our healthy subjects in response to small intestinal nutrients is intriguing. This was a consistent observation in all the 12 healthy subjects. Physiologically, it is possible that the proximal gastric volume reduction in the middle of the infusion represents proximal gastric contractions to redistribute feed to the distal stomach. This pattern of proximal gastric response, however, has not previously been reported in barostat studies during intra-gastric^[22,23] or intra-duodenal^[36] nutrient delivery. In the Barbara *et al*^[36] study, intra-gastric volume recording was only performed for 30 min during duodenal nutrient infusion, which may have been too short to detect the second peak. Although the reasons for differences in the proximal gastric response reported to date remain unclear, they may relate to the site of nutrient administration and duration of infusion.

There were a number of issues that should be acknowledged in the current study. Intra-gastric delivery of nutrient was avoided for several reasons. Firstly, the frequently observed slow gastric emptying in this group of patients^[6] may lead to an unreliable assessment of the feed-back response, as a constant delivery of 2-3 kcal/min of nutrient to the small intestine is required^[9,10]. Secondly, gastric motility and emptying of a liquid meal can be altered by the presence of a barostat balloon, which redistributes the meal to the distal stomach, increases intra-gastric pressure and accelerates gastric emptying, with 50% of the liquid meal emptying in the first 10 min^[22]. Thereafter, the rate of emptying is dependent on the phasic-tonic contraction of the proximal stomach. In addition, although the effects of performing endoscopy on gastric motility are unknown, they are unlikely to be important in the context of nasogastric tube intubation. The procedure was performed using a skinny endoscope with minimal air inflation and all gastric contents were aspirated from the stomach immediately prior to the study. Barostat studies are best performed in the sitting, upright position^[19], but it is impossible to study critically ill patients in this posture. The current study was thus performed

in a 30 degree recumbent position in both patients and healthy subjects, to minimize the effects of posture on bag volume. Hebbard *et al*^[37] recommended that if upright posture is not possible, then the same posture should be controlled between the study groups. In addition, proximal gastric compliance was not assessed. Although this would be of major interest, it was not performed because of concern that positive intra-bag pressures would potentially interfere with patient ventilation, due to splinting of the diaphragm. Finally, although our patients were significantly older, we do not believe this would substantially affect the study results, as healthy aging does not affect proximal gastric motor responses to a meal^[38].

In conclusion, the proximal gastric response to small intestinal nutrients is abnormal in critical illness, characterised by a prolonged relaxation after nutrient stimulation and a reduction in fundic wave activity. The potential contribution of these proximal gastric motor abnormalities to delayed gastric emptying in critically ill patients requires further study.

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Effect of shenfu injection on gastrointestinal microcirculation in rabbits after myocardial ischemia-reperfusion injury

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Abstract

AIM: To investigate the effect of shenfu injection on gastrointestinal microcirculation after myocardial ischemic-reperfusion (IR) injury in rabbits and probe into the mechanism.

METHODS: Forty healthy flap-eared white rabbits were randomly divided into 4 groups: IR injury control group (group I), shenfu injection 5 mL/kg per h group (group II), shenfu injection 10 mL/kg per h group (group III) and shenfu injection 20 mL/kg per h group (group IV). The four groups were treated with Lactated Ringer's solution, shenfu injection 5, 10, and 20 mL/kg per h were infused intravenously 30 min before experiment respectively. The values of hemodynamics [mean arterial pressure (MAP), heart rate (HR), gastric intramucosal partial pressure of carbon dioxide (PCO₂), blood gas analysis and pH] were measured and compared with those before myocardial ischemia, 60 min after myocardial ischemia and 60, 90, and 180 min after reperfusion.

RESULTS: The MAP, HR and gastric intramucosal pH were (70.50 ± 4.50) kPa, (165 ± 14) beats per min, 7.032 ± 0.024 in group I 60 min after myocardial ischemia, which were significantly decreased compared with those before myocardial ischemia (88.50 ± 9.75 kPa, 217 ± 18 beats per min, 7.112 ± 0.035, *P* < 0.05). The MAP, HR and gastric intramucosal pH were significantly decreased in group I 60, 90, and 180 min after reperfusion (61.50 ± 5.25 kPa, 133 ± 31 beats per min, 6.997 ± 0.025) compared with those before reperfusion respectively (*P* < 0.05), whereas the values were insignificantly different in groups II, III or IV after reperfusion, compared with those before reperfusion, and there were no significant differences between groups II, III, and IV after reperfusion.

CONCLUSION: Pre-infusion of shenfu injection has a

protective effect on gastrointestinal microcirculation after myocardial IR injury in rabbits, in a dose independent manner.

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Key words: Shenfu injection; Myocardial ischemic-reperfusion injury; Gastrointestinal microcirculation

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INTRODUCTION

Ischemia-reperfusion injury is a significant etiological factor of organ dysfunction, and it is important to investigate protective measures for anti-ischemia reperfusion injury. Myocardial ischemia reperfusion injury can induce myocardial metabolic disturbance, organ and functional damage, hemodynamic change, cardiac output decrease, reduction of gastrointestinal tract blood flow and oxygen dysmetabolism^[1]. The gastrointestinal mucosa has rich blood supply, being fairly sensitive to ischemia and oxygen deficiency, and is usually involved first after hypoxia. It is one of internal organs that have decrease of blood flow early and obviously but recover lastly. In this study, we investigated the effect of shenfu injection on gastrointestinal microcirculation after myocardial ischemic-reperfusion injury and probed into the mechanism of protective effect of shenfu injection through animal experiments in rabbits. Shenfu injection is a traditional Chinese medicine. The main pharmaceutical compounds are shenfu and fuzi.

MATERIALS AND METHODS

Materials

This was a random controlled animal experimental study carried out in the Institute of Cardiology and Anesthesiology of Qingdao Medical University. Forty healthy flap-eared white rabbits were provided by Animal Centre of Affiliated Hospital of Qingdao Medical University [certificate number: FYXK (LU20030035)], including males and females, weighing 2.3-3.1 kg, with a

mean 2.46 ± 0.29 kg. They were fed with entire nutrition forage. Every rabbit ate about 150 g of foodstuffs, twice a day with free access to water. Rabbits were housed in an airy, quiet and light room with room temperature at 20°C - 22°C , one in each cage. Forty rabbits were randomly divided into 4 groups with 10 rabbits in a group, including ischemia-reperfusion control group (group I); shenfu injection 5 mL/kg per h group (group II); shenfu injection 10 mL/kg per h group (group III); shenfu injection 20 mL/kg per h group (group IV). Rabbits were fasted for 12 h before experiment. There was no significant difference in weight between four groups.

Methods

After being anaesthetized by an intravenous injection of thiopental sodium (20 g/L) with a dosage of 25 mg/kg, rabbits were incised at trachea, and an endotracheal catheter was inserted and connected to an animal breathing apparatus with 100% inhaling oxygen, 10-13 mL/kg tidal volume, 20-30 breath frequency per min and 1.2 inspiration/expiration ratio. Pressure of carbon dioxide at the end of expiration was kept at 4.7-5.3 kPa. A tube was inserted into right internal carotid vein for infusion of fluid and shenfu injection; an arterial puncture needle was inserted into the femoral artery and connected to a pressure-energy transducer to monitor blood pressure, heart rate and sample blood while mean artery pressure was kept between 69.75-90.00 kPa. Meanwhile, a 14F TRIP Tonometry catheter was inserted into stomach. During the experiment fentanyl (25 $\mu\text{g}/\text{kg}$) and vecuronium bromide (0.1 mg/kg) were administrated to maintain anaesthesia. We did not record baseline values until blood pressure, heart rate and breath were stable for 10 min while calculating pH according to Henderson-Hasselbalch formula^[2]. Groups I, II, III, and IV were infused with natrium lacticum Ringer's solution 8 mL/kg per h, shenfu injection 5 mL/kg per h, shenfu injection 10 mL/kg per h and shenfu injection 20 mL/kg per h through the vein respectively 30 min before the model of myocardial ischemia reperfusion was established. The pericardium was unfolded after chest was opened and the heart was exposed thoroughly. The left coronary artery anterior descending branch (LAD) was identified and main branch ligated at mid or upper 1/3 with double clinch. The ligature was unclamped for reperfusion after ischemia for 60 min.

Main observation index

Baseline levels of mean artery pressure, heart rate and pH value, at myocardial ischemia for 60 min and after reperfusion for 60, 90, and 180 min were recorded respectively.

Statistical analysis

All data were analyzed with SPSS 10.0 statistical package. Data were expressed as mean \pm SD, analyzed with Student's *t*-test between intra-group and with ANOVA between groups. A value of $P < 0.05$ was considered to have statistical significance.

Table 1 Physiologic indexes after myocardial ischemia-reperfusion injury (mean \pm SD, $n = 10$)

Group	Mean artery pressure (kPa)	Heart rate (beats/min)	pH value
Baseline level			
Group I	95.25 \pm 6.00	206 \pm 13	7.129 \pm 0.038
Group II	88.50 \pm 9.75	217 \pm 18	7.112 \pm 0.035
Group III	86.25 \pm 8.25	198 \pm 15	7.131 \pm 0.045
Group IV	90.75 \pm 6.75	221 \pm 22	7.122 \pm 0.037
Ischemia 60 min			
Group I	70.50 \pm 4.50 ^a	165 \pm 14 ^a	7.032 \pm 0.024 ^a
Group II	87.00 \pm 10.50	201 \pm 17	7.108 \pm 0.023
Group III	84.75 \pm 4.50	195 \pm 20	7.125 \pm 0.031
Group IV	87.75 \pm 5.25	213 \pm 16	7.119 \pm 0.026
Reperfusion 60 min			
Group I	61.50 \pm 5.25 ^{a,c}	133 \pm 31 ^{a,c}	6.997 \pm 0.025 ^{a,c}
Group II	85.50 \pm 9.00	197 \pm 12	7.121 \pm 0.022
Group III	96.75 \pm 6.00	199 \pm 16	7.114 \pm 0.032
Group IV	94.50 \pm 12.00	208 \pm 13	7.126 \pm 0.019
Reperfusion 90 min			
Group I	57.75 \pm 6.00 ^{a,c}	119 \pm 25 ^{a,c}	6.953 \pm 0.019 ^{a,c}
Group II	81.75 \pm 10.50	210 \pm 22	7.118 \pm 0.033
Group III	84.00 \pm 6.75	205 \pm 17	7.128 \pm 0.027
Group IV	81.00 \pm 11.25	214 \pm 21	7.125 \pm 0.026
Reperfusion 180 min			
Group I	48.75 \pm 3.00 ^{a,c}	94 \pm 16 ^{a,c}	6.911 \pm 0.017 ^{a,c}
Group II	86.25 \pm 5.25	204 \pm 25	7.127 \pm 0.024
Group III	90.75 \pm 9.00	216 \pm 21	7.132 \pm 0.034
Group IV	85.50 \pm 4.50	200 \pm 18	7.120 \pm 0.029

Group I: Ischemia-reperfusion control group; group II: Shenfu injection 5 mL/kg per h; group III: Shenfu injection 10 mL/kg per h; group IV: Shenfu injection 20 mL/kg per h. ^a $P < 0.05$ vs baseline level; ^c $P < 0.05$ vs ischemia 60 min.

RESULTS

None of the 40 rabbits died during experiment. Hemodynamics index and pH value changes of stomach mucosa are shown in Table 1.

DISCUSSION

Studies show^[3] that myocardial IR injury often induces gastric intramucosal ischemia and oxygen deficiency. The gastrointestinal microcirculation is very sensitive to ischemia and oxygen deficiency and the gastrointestinal mucous membrane is a perfect region for supervision, while the pH value of gastric intramucosa (Phi) has become a regional index for tissue perfusion. In recent years, the pH value of gastric intramucosa has been a sensitive index to reflect gastric intramucosal ischemia and oxygen deficiency^[4]. The main effective ingredients of shenfu injection are panaxoside and myoctonine^[5]. According to the literature, panaxoside not only has an important role in antistress, antioxygenation and myocardial ischemia, but also has definite protective and therapeutic activity against myocardial ischemia injury, renal ischemia reperfusion injury, and spinal cord ischemia injury^[6,7]. However, reports about its effect on gastrointestinal micro-circulation are rare. The current study showed that the mean arterial blood pressure and heart rate in group I decreased progressively, both

were lower than that in baseline condition ($P < 0.05$) in myocardial reperfusion model when myocardial ischemia lasted for 60 min, or after reperfusion for 60, 90, 180 min. However, those indexes of three shenfu groups were not significantly changed ($P > 0.05$) when myocardial ischemia lasted for 60 min, or after reperfusion for 60, 90, 180 min, and there were no significant differences between three shenfu groups. Therefore we suppose that hemodynamics were unstable after myocardial ischemia reperfusion, which induced the decrease of blood pressure and heart rate. On the other hand, shenfu injection could improve the hemodynamics during myocardial ischemia reperfusion, and the protective effect was not significantly dose dependent. Moreover, pH value of group I decreased significantly compared with that of baseline level ($P < 0.05$). The pH value of shenfu injection therapeutic groups II, III, IV did not change ($P > 0.05$), which proved that shenfu injection could improve and prevent the hypoperfusion and disturbance of oxygenation in gastrointestinal microcirculation after myocardial ischemia and reperfusion, and has an important protective role in gastrointestinal microcirculation. We designed 3 dose groups: 5, 10, and 20 mL/kg per h and discovered that the pH value in different groups was not changed significantly ($P > 0.05$). This showed that pre-infusion of shenfu injection had an effective protective function on gastrointestinal microcirculation after myocardial IR injury in rabbits, in a dose independent manner. After myocardial ischemia reperfusion, because of hemodynamic instability, gastrointestinal tract hypoperfusion, oxygenation disorder, generation of free radicals and the role of cytokines, activation and releasing of mediators of inflammation, gastrointestinal microcirculation was disturbed^[8,9], leading to gastric intramucosal ischemia, hypoxia, and pH decrease. The protection of shenfu injection on gastrointestinal microcirculation is associated with many factors^[10,11], such as myocardial preservation, stabilization of hemodynamics, antioxygenation, reduced production of free radicals, degradation of cytokines and inhibition of the activation and release of mediators of inflammation.

In summary, myocardial ischemia reperfusion injury can induce gastrointestinal microcirculation dysfunction.

Shenfu injection has protective effect on gastrointestinal microcirculation after myocardial IR injury in rabbits, with no obvious dose-effect relationship. Shenfu injection may improve gastrointestinal microcirculation mainly through myocardial preservation, and stabilization of hemodynamics. Our study provides strong evidence for the protective effect of shenfu injection against IR injury.

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RAPID COMMUNICATION

Is leptin related to systemic inflammatory response in acute pancreatitis?

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Abstract

AIM: To evaluate the relationship between leptin and systemic inflammation in acute pancreatitis.

METHODS: Consecutive patients with acute pancreatitis were included. Body mass index and serum samples were obtained at admission. Leptin, TNF- α , IL-6, -8 and -10 levels were determined by ELISA. Severity was defined according to Atlanta criteria.

RESULTS: Fifty-two (29 females) patients were studied. Overall body mass index was similar between mild and severe cases, although women with severe pancreatitis had lower body mass index ($P = 0.04$) and men showed higher body mass index ($P = 0.05$). No difference was found in leptin levels regarding the severity of pancreatitis, but higher levels tended to appear in male patients with increased body mass index and severe pancreatitis ($P = 0.1$). A multivariate analysis showed no association between leptin levels and severity. The strongest cytokine associated with severity was IL-6. Correlations of leptin with another cytokines only showed a trend for IL-8 ($P = 0.058$).

CONCLUSION: High body mass index was associated with severity only in males, which may be related to android fat distribution. Serum leptin seems not to play

a role on the systemic inflammatory response in acute pancreatitis and its association with severe outcome in males might represent a marker of increased adiposity.

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Key words: Leptin; Cytokines; Obesity; Acute pancreatitis; Prognosis

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INTRODUCTION

Obesity is a risk factor for severe acute pancreatitis (AP) and for the development of both local and systemic complications^[1]. A chronic, low grade, pro-inflammatory state has been described in human obesity, including increased plasma levels of IL-6, TNF- α , C-reactive protein and endothelial adhesion molecules^[2]. This may be due to the cytokine-synthesizing capabilities of adipose tissue driven by yet unknown signals, giving rise to insulin resistance and the metabolic syndrome^[3]. Actually, one third of the circulating IL-6 originates from adipose tissue, especially from the visceral depot^[4]. On the other side, the inflammatory cascade evoked by AP has a pivotal role in determining severity of the disease, and many cytokines and other acute reactants are well-established prognostic markers (i.e. IL-6, -8, -10, C reactive protein)^[5]. Coincidence of both a chronic and acute inflammatory scenarios (obesity and AP, respectively) with similar cytokine patterns may offer an explanation for the association between severe AP and obesity. Therefore, obese individuals may be prone to develop a more intense inflammatory reaction in response to AP that would lead to a dismal prognosis.

Leptin, the protein derived from the *Ob* gene, is an adipocyte-derived signal^[6], and its circulating concentrations are highly correlated with body fat mass^[7,8]. It has also been implied as a modulator of immunity with pro-inflammatory properties^[8,9], and it is highly correlated to C-reactive protein, independently of obesity^[10].

Research about leptin in AP is scarce. Two experimental

studies in animal models have suggested a protective role for leptin on AP by suppression of the pro-inflammatory response, reduction of leukocyte infiltration on pancreatic tissue and increased pancreatic repair^[11,12]. These results contrast with those found when studying non-pancreatic conditions, suggesting a tissue-specific protective role for leptin. Also, elevated plasma levels of leptin were found in patients with AP when compared to healthy controls with similar body mass index^[11]. However, distinction between severe and mild cases was not established.

Whether circulating leptin would have a pro- or anti-inflammatory role in AP, its serum concentrations should correlate with severity in AP, as has been reported for IL-6 and IL-10, respectively. It should also correlate with other cytokines previously found to be increased in severe AP (i.e. TNF- α , IL-6, -8, -10). The purpose of the present work was to measure serum concentrations of leptin and other cytokines in patients with severe and mild AP to identify a possible role of the former in the systemic inflammatory response, and in the prognosis assessment of AP.

MATERIALS AND METHODS

All patients with AP diagnosis admitted between March 2002 and August 2004 to a tertiary medical center were included in the study. Diagnosis of AP was based on typical clinical manifestations with at least a 3-fold increase of serum amylase and/or lipase. Whenever uncertainty about diagnosis existed, CT-scan was performed to confirm/rule out AP. Patients were excluded if they had received IV therapy before admission. HIV/AIDS patients were also excluded because of the confounding lipodystrophy and altered leptin levels described in these patients^[13].

The etiology of AP was classified as biliary, alcoholic, idiopathic or other. Weight and height from all patients were measured during the first 48 h from admission for body mass index (BMI) calculation. Prognostic severity by modified Ranson's criteria was recorded for each patient^[14].

Severe AP was considered when patients developed one or more local (i.e. necrosis, infected necrosis, abscess, pseudocyst) or systemic (i.e. renal or respiratory failure, cardiovascular collapse, coagulopathy, gastrointestinal bleeding, sepsis or multiple organ failure) complications according to the Atlanta classification of AP^[15]. Patients were managed by the medical house staff who were blinded as to the aims and methodology of this study.

During the first 24 h from admission, aliquots of a serum sample obtained from all patients were stored at -70°C, until laboratory determinations were performed. Serum leptin levels were measured in duplicate in the same run using a commercial ELISA kit for human leptin (Linco Research, MO, USA), with a sensitivity of 0.5 ng/mL. According to protocol insert, mean serum leptin levels from normal individuals (BMI: 18-25 kg/m²) are 3.8 \pm 1.8 ng/mL for men, and 7.4 \pm 3.7 ng/mL for women. TNF- α , IL-6, -8 and -10 concentrations were quantified using commercially available ELISA kits (Beckton Dickinson, NL, USA), according to the manufacturer's instructions.

BMI was expressed as mean \pm SD, while serum de-

Table 1 Body mass index (BMI) and leptin levels in acute pancreatitis (AP) according to severity status and gender

	<i>n</i>	BMI	BMI \geq 25	BMI \geq 30	Leptin (ng/mL)
		mean \pm SD	<i>n</i> (%)	<i>n</i> (%)	Median (range)
All patients	52	27 \pm 5	32 (63)	13 (25)	8.6 (0.5-215)
Severe AP	14	27 \pm 5	7 (54)	2 (15)	10.3 (0.5-61)
Mild AP	38	27 \pm 5	25 (66)	11 (29)	7.7 (0.5-215)
Women	29	27 \pm 5	18 (64)	9 (32)	11 (0.5-215) ^a
Men	23	27 \pm 4	14 (61)	4 (17)	3.7 (0.5-32)
Women & severe AP	7	24 \pm 3 ^b	2 (33)	0	10.4 (1.39 - 61)
Women & mild AP	22	28 \pm 6	16 (73)	9 (41)	11.7 (0.5-215)
Men & severe AP	7	29 \pm 6 ^c	5 (71)	2 (29)	7 (0.5-32) ^d
Men & mild AP	16	26 \pm 3	9 (65)	2 (13)	3.4 (0.5-19)

^a*P* = 0.008, ^b*P* = 0.04, ^c*P* = 0.05, ^d*P* = 0.1 (Mann-Whitney U-test).

terminations were expressed as medians and ranges. Comparisons were carried out by means of the *t* test and Mann-Whitney U-test, as appropriate. A multivariate analysis was constructed to test the independent association of circulating leptin and severity, adjusted for potential confounders (gender and BMI). Correlations among cytokines and any other non-parametric variable were done using the Spearman rank-order test. All statistical analysis was performed using SPSS statistical software (Chicago, Illinois, USA). *P* < 0.05 was considered statistically significant.

Informed consent was obtained from all patients. This study was approved by the local Institutional Board for Human Research and designed according to international guidelines.

RESULTS

A total of 58 patients with AP were studied. Six patients were excluded from the analysis, four of them because of HIV/AIDS and two because of unavailability of serum for complete leptin and/or cytokines determinations (2 patients had severe AP, 33%).

There were 29 women (56%) and mean age of all patients was 42 \pm 16 years. The most frequent AP etiology was biliary (*n* = 19), followed by hypertriglyceridemia (*n* = 7), alcoholic (*n* = 6) and post-ERCP (*n* = 4). Other causes included: acute episodes of chronic pancreatitis (*n* = 4), pancreas divisum (*n* = 2), drug-induced (*n* = 2) and hypercalcemia (*n* = 1); while 7 cases were considered idiopathic. First time AP was present in 31 patients (60%). Severity was identified in 14 cases (27%). Local complications occurred in 8 (15%) patients, whereas systemic complications developed in 11 (21%); presence of both complications was noted only in 5 cases. Six patients (12%) were admitted to the ICU and 4 had lethal AP (8%).

BMI according to severity status and gender is shown in Table 1. Although there was no difference between severe and mild cases in all patients, females with severe AP had significantly lower BMI when compared to mild

Table 2 Independent association between leptin levels and severity of acute pancreatitis (AP) adjusted by gender and body mass index (BMI)

	Overall	Gender adjusted	BMI adjusted
Leptin and AP severity	$P = 0.766$	$P = 0.5$	$P = 0.272$

Table 3 Levels of cytokines (other than leptin) in acute pancreatitis (AP) according to severity status and gender

	All patients				Women			Men		
	All patients	Severe AP	Mild AP	<i>P</i>	Severe AP	Mild AP	<i>P</i>	Severe AP	Mild AP	<i>P</i>
¹ TNF- α	0 (0-579)	13 (0-385)	0 (0-579)	0.021	30 (0-385)	0 (0-121)	0.036	2 (0-89)	1 (0-579)	NS
¹ IL-6	52 (1-560)	206 (10-590)	40 (1-537)	< 0.001	131 (10-361)	40 (1-180)	0.013	298 (195-590)	42 (3-537)	< 0.001
¹ IL-8	4 (0-4596)	99 (0-4596)	4 (0-1322)	NS	4 (0-780)	4 (0-496)	NS	182 (0-4596)	4 (0-1322)	NS
¹ IL-10	4 (0-560)	6 (0-54)	2 (0-560)	0.028	4 (0-17)	0 (0-49)	NS	27 (4-54)	4 (0-560)	NS

¹ pg/mL, all results expressed as median (range).

Table 4 Leptin correlation with other cytokines according to gender

		TNF- α	IL-6	IL-8	IL-10
		<i>r</i> (<i>P</i>)	<i>r</i> (<i>P</i>)	<i>r</i> (<i>P</i>)	<i>r</i> (<i>P</i>)
Leptin	All patients	NS	NS	0.265 (0.058)	NS
	Females	NS	NS	0.488 (0.007)	NS
	Males	0.487 (0.019)	0.397 (0.061)	NS	NS

cases; while an opposite association was observed in male patients. There was a positive correlation of BMI and leptin ($r = 0.476$, $P < 0.001$) in the whole group. Leptin levels were significantly higher in women when compared with men and a tendency for higher leptin levels was demonstrated in severe male cases. After multivariate analysis, there was no significant independent association between leptin serum levels and severity of AP, neither in the overall sample, nor after adjusting by gender and BMI (Table 2). There was no difference in leptin measurements between patients admitted or not to the ICU, or between a fatal outcome or not ($P = 0.41$ and $P = 0.34$, respectively). Time of evolution from onset of pain (1.7 ± 2 d) did not alter leptin values significantly (data not shown).

Predicted severity by modified Ranson's criteria correlated with Atlanta criteria ($r = 0.414$, $P = 0.002$), was significantly associated with increased levels of TNF- α ($P = 0.018$) and IL-8 ($P = 0.021$), and showed a tendency for increased IL-6 ($P = 0.096$); however, it did not correlate with leptin levels.

Levels of cytokines measured according to severity status (Atlanta criteria) and gender are shown in Table 3. TNF- α , IL-6 and IL-10 were higher in the group with severe AP. In the sub-analysis by gender, IL-6 was the only marker to remain higher in severe cases. Local complications were associated with higher median [range] levels of IL-6 [250 (9.5-590) *vs* 47 (0.7-537), $P = 0.004$], while systemic had increased levels of IL-6 [202 (62-590)

vs 40 (0.7-537), $P < 0.001$] and IL-10 [7.2 (0-54) *vs* 2.7 (0-560), $P = 0.025$]. Admission to the ICU was associated with elevated IL-6 [235 (62-590) *vs* 47 (0.7-537), $P = 0.006$] and IL-8 [258 (3.5-4596) *vs* 3.5 (0-1322), $P = 0.036$], and mortality was related with higher levels of TNF- α [75 (1.6-385) *vs* 0 (0-579), $P = 0.015$] and IL-8 [538 (269-4596) *vs* 3.5 (0-1322), $P = 0.005$].

In the whole group, leptin did not correlate significantly with any other cytokine, although a tendency was noted with IL-8 (Table 4). When only females were taken into account the latter resulted significant, whereas if only males were analyzed, leptin correlated significantly with TNF- α and tended to correlate with IL-6. However, TNF- α was significantly correlated with IL-8 ($r = 0.379$, $P = 0.006$) and IL-10 ($r = 0.317$, $P = 0.023$), IL-6 with IL-10 ($r = 0.412$, $P = 0.002$), and IL-8 with IL-10 ($r = 0.275$, $P = 0.048$). There was also a tendency for TNF- α with IL-6 ($r = 0.231$, $P = 0.1$).

DISCUSSION

Severe AP has a mortality around 24%-50%^[5,16]. Therefore, major efforts have focused on the discovery of prognostic markers for an early identification of severe cases, to anticipate a more aggressive diagnostic and therapeutic approach. The recognition of obesity as a risk factor for severe AP^[11] lead to the identification of other markers of obesity, such as android fat distribution and higher waist circumference, as more strongly associated with severity^[17]. These findings provided a possible link between AP and the so-called metabolic syndrome, which among certain criteria is characterized by a chronic pro-inflammatory condition^[3].

Particularly since the identification of human leptin in 1994, adipose tissue has been increasingly recognized as an endocrine organ capable of coordinating a variety of biological processes including not only energy metabolism, but neuroendocrine and immune functions^[14]. IL-6, TNF- α and leptin are among the principal immune-related

signals produced by adipose tissue^[3,4]. *In vitro* and *in vivo* experimental studies have shown that leptin is capable of promoting neutrophils activation and chemotaxis, inducing TNF- α and IL-6 from monocytes, promoting monocytes/macrophages activation and phagocytosis, enhancing lymphocytes proliferation and favouring a Th1 cytokine response^[8,18]. Also, it has been demonstrated that leptin can restore immune function under critical conditions such as starvation and it has been linked to autoimmunity^[8,9]. Consequently, adipose tissue and leptin seem to play a major role on immunological status, being the latter a pro-inflammatory signal.

On the pancreatic view, TNF- α , IL-1, -8, -10, and especially IL-6, have a major role on the pathophysiology of AP, as part of the inflammatory network evoked systemically^[5]. Experimental studies of AP in rats have shown that leptin levels increase after cerulein-induced pancreatitis, presumably from pancreatic origin (increase in leptin mRNA from the pancreas), and that exogenous administration is associated with diminished histological manifestations of pancreatitis and reduced plasma TNF- α ^[11]. Similarly, administration of leptin reduced the severity of ischemia/reperfusion-induced pancreatitis by diminishing morphological features of pancreatic damage and serum IL-1 β levels, while accelerated pancreatic repair by increasing pancreatic blood flow and DNA synthesis^[12]. These results are contrary to what would be expected according to other experimental studies evaluating the effect of leptin in non-pancreatic tissues^[8,9,18], and point-out a possible tissue-specific anti-inflammatory role in AP.

The absence of relationship between BMI and severity in the present study may be related to our reduced sample size, or to the predominance of female patients. Most reports on obesity as a prognostic marker of AP (including one meta-analysis) have not addressed whether this association may be modified by gender. However, two previous studies by our group have found that the association between obesity and severity is mostly seen in male patients^[17,19]. A clear explanation for this dichotomy on the risk of obesity on AP is unavailable yet, although it may be related to the higher waist to hip ratio or waist circumference (android fat distribution) found in males^[17].

Our results on the positive correlation of leptin with BMI, as well as the higher levels in females are in agreement with what has been previously described^[6]. Leptin levels did not differ between severe and mild cases, although male patients with severe AP had higher leptin levels in association with increased BMI, which may only represent a marker of increased adiposity^[6]. Moreover, these results were supported by the lack of association between leptin levels and severity in a multivariate analysis adjusted for gender and BMI. Similar findings occurred in patients with inflammatory bowel disease in whom leptin differences were entirely explained by variations in BMI, rather than disease activity^[20]. Furthermore, leptin did not correlate with any other cytokine in the whole group, while in the correlation by gender, leptin was neither correlated with the cytokines increased in severe cases (TNF- α and IL-6 for females, IL-6 and IL-8 for males, IL-10 for both). Although there was a positive association between TNF- α and leptin levels for males, all the evidences above make

it difficult to think that leptin plays a major role in the inflammatory response of AP; whether as a pro- or anti-inflammatory cytokine.

TNF- α , IL-6 and IL-10 were increased in the whole group of patients with severe AP, although IL-6 was the only cytokine that remained significantly higher in women and men with severe episodes, either in presence of local or systemic complications, or when admitted to the ICU. However, mortality was associated with elevation in serum levels of TNF- α and IL-8. Both cytokines, TNF- α and IL-8, were associated with Ranson's prognostic scale for mortality. Many studies have consolidated IL-6 as the cytokine with the most important prognostic utility for severity within the initial 24 h, although it seems not to be useful for prediction of death^[5,21-23]. This cytokine has also demonstrated use for identification of infected necrosis^[24]. Similarly, IL-8, TNF- α and IL-10 have early prognostic utility in AP: all of them are increased early in severe cases^[22,25] and IL-8 has been previously found increased in fatal AP^[26].

Konturek *et al.* found that leptin levels were increased in patients with AP when compared to BMI- and age-matched controls, however this study did not distinguish mild from severe cases^[11]. It is well known that acute inflammatory conditions (i.e. AP) are associated with hyperglycemia, insulin resistance and increased levels of leptin, IL-6 and TNF- α ^[3]; and it is also recognized that insulin resistance leads to leptin resistance and further elevation of leptin concentrations^[27]. Therefore, this could be the explanation for previously reported increased leptin levels in AP that would result in even further increased levels if superimposed on a chronic inflammatory condition (i.e. obesity), as was observed in male patients with AP and higher BMI. Since our findings do not support leptin as being associated with a more severe inflammatory response and worse prognosis (i.e. severity, mortality or admission to ICU), it could represent just a bystander of the inflammatory reaction in AP; and unlike IL-6, IL-10 and TNF- α , not play a role on its pathophysiology.

The role of leptin as an anti-inflammatory and protective signal as was demonstrated by some experimental models in rats^[11,12], should have lead to significantly increased or decreased levels in severe cases, which was not demonstrated in our results; therefore, not ascertaining leptin as a protective signal in AP. This difference could only represent species differences in the actions for leptin, as has been shown with respect to leptin effects on glucocorticoids (these are decreased by leptin in mice, while not affected in humans)^[8].

In summary, our results do not support human leptin as a major pro-inflammatory signal involved in AP, nor as a protective and anti-inflammatory mediator. It seems neither to be the link between obesity and a higher rate of complications in AP; nor a prognostic marker.

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A functional variant in the CD209 promoter is associated with DQ2-negative celiac disease in the Spanish population

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HLA-DQ2 (-) group (carrier A vs GG in DQ2 (-) vs DQ2 (+) patients ($P = 0.026$, OR = 3.71).

CONCLUSION: The -336G *CD209* allele seems to be involved in CD susceptibility in HLA-DQ2 (-) patients. Our results might suggest a possible role of pathogens in the onset of a minor group of CD patients.

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Key words: CD209; HLA-DQ2; Celiac disease; Single nucleotide polymorphism; Susceptibility

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Abstract

AIM: To address the role of *CD209* in celiac disease (CD) patients. Non-human leukocyte antigen (HLA) genetic factors in CD predisposition are poorly understood, and environmental factors like infectious pathogens may play a role. *CD209* is a dendritic and macrophage surface molecule involved in pathogen recognition and immune activation. Recently, a functional variant in the promoter of the *CD209* gene (-336A/G) has been shown to affect the transcriptional *CD209* activity *in vitro* and it has been associated with a higher susceptibility to/or severity of infection.

METHODS: The study population was composed of two case-control cohorts of 103 and 386 CD patients and 312 y 419 healthy controls as well as a panel of 257 celiac families. Genotyping for the -336A/G *CD209* promoter polymorphism was performed using a TaqMan 5' allelic discrimination assay. HLA-DQ was determined by hybridization with allele specific probes.

RESULTS: Initially, the case-control and familial studies did not find any association of the -336 A/G *CD209* genetic variant with CD susceptibility. However, the stratification by HLA-DQ2 did reveal a significant association of *CD209* promoter polymorphism in the

INTRODUCTION

Celiac disease (CD) is a chronic inflammatory disease with a multi-factorial origin. Genetically susceptible individuals show a pathological inflammatory response after exposure to gluten, a protein present in wheat, barley, rye and oats^[1]. Genetic studies in CD have revealed a strong influence of human leukocyte antigen (HLA) class II genes, specifically the alleles DQA1*0501 and DQB1*02 (HLA-DQ2) and to a lesser extent DQA1*0301 and DQB1*0302 (HLA-DQ8). However, other genes outside the HLA region must be also involved in CD susceptibility since the genetic susceptibility to CD indicated by the λ_s (sibling risk/population frequency) is around 30%-60%, but no more than 40% to that sibling familial risk has been attributed to HLA^[2,3].

CD can be considered a model of autoimmune diseases, being for the moment the only one in which the environmental factor that triggers the inflammatory response (gluten) has been clearly identified^[4]. Nevertheless, it is suggested that other environmental factors may affect the disease onset. It has been speculated that certain viruses or pathogens may somehow alter the tolerance in the intestinal mucosa, or induce an inflammatory state in the intestinal mucosa, prone to react against gluten-derived

peptides^[5]. This additional environmental input might be of higher importance in those patients with low genetic susceptibility (e.g., patients without HLA-DQ2). The interaction between the pathogen and gut immune cells might be an essential mechanism in this pathway. In this regard, an important mediator of pathogen recognition by dendritic cells is CD209 or DC-SIGN (dendritic cell specific intercellular adhesion molecule 3 grabbing non-integrin), encoded by the *CD209* gene. CD209 is a type II transmembrane protein present in dendritic cells and macrophages and a member of the C-type lectin receptor family. Natural ligands of CD209 include self-molecules, ICAM-2 and ICAM-3, but it also binds to pathogens and pathogen-derived molecules. This interaction may be used by some pathogens as an immunoinvasive strategy through binding and internalization, although always as one among other alternative ways^[6]. Furthermore, the *CD209* gene is located within the 19p13.1 chromosomal region, showing a strong linkage with CD susceptibility in a genome-wide study^[7].

In the *CD209* promoter region, a putative functional variant (-336A/G; rs4804803) affecting a Sp1-like binding site has been described^[8]. This single nucleotide polymorphism (SNP) can affect the transcriptional *CD209* activity *in vitro* and is associated with a higher susceptibility to HIV-1 infection and a higher dengue fever severity.

On this basis, we aimed to investigate the possible involvement of *CD209* -336 genetic variant in CD susceptibility.

MATERIALS AND METHODS

Subjects

A total of 103 celiac disease patients and 312 ethnically matched healthy controls recruited from the Hospital Virgen de las Nieves (Granada, Spain) were initially studied to assess the influence of the -336A/G *CD209* polymorphism in celiac disease. Subsequently, a second larger cohort recruited from the Hospital Clínico San Carlos and Hospital La Paz (Madrid, Spain) and consisting of 386 celiac patients and 419 healthy controls was analyzed to obtain more conclusive results. The age at study of all patients was 7.1 ± 3.9 years, and the mean age for diagnosis was 2.7 ± 2.72 years. A total of 60% were women. We extended our study to a panel of 257 pairs of progenitors of the CD patients studied, specifically 103 families from Granada and 154 from Madrid were obtained, all of them composed of an affected child and their parents.

The participants from both the familial and case-control analyses were of Spanish white origin. All the samples were collected after informed consent was obtained. Celiac disease patients were diagnosed following the European Society for Paediatric Gastroenterology and Nutrition (ESPGAN) criteria^[9]. This study was approved by the ethics committee of the respective hospitals.

Genotyping

Taqman 5' allelic discrimination assay was performed to genotype the *CD209* -336 genetic variant. Primers

Table 1 Genotypic and allelic CD209 frequencies in celiac patients ($n = 103$) and controls ($n = 312$) from the Granada area

Genotype	Celiac patients <i>n</i> (%)	Controls <i>n</i> (%)	<i>P</i>
AA	61 (59)	191 (61)	
AG	36 (35)	109 (35)	
GG	6 (6)	12 (4)	0.7
Allele			
A	158 (77)	491 (78)	
G	48 (23)	133 (21)	0.6

and probes were provided by Custom-Taqman-SNP-Genotyping-Assay Service (Applied Biosystems, Foster City, CA, USA). The primer sequences are 5' - GGACAGTGCTTCCAGGA ACT -3' (sense) and 5' - TGTGTTACACCCCTCCACTAG -3' (antisense). The sequences of Taqman MGB probes are 5'-TACCTGCCTACCCTTG-3' and 5'-CTGCCACCCCTTG-3'. The probes were labeled with the fluorescent dyes VIC and FAM, respectively. Polymerase chain reaction (PCR) was carried out in a total volume of 12.5 l using the following amplification protocol: denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 92°C for 15 s, and annealing and extension at 60°C for 1 min. Then genotype of each sample was attributed by measuring the allele-specific fluorescence in the ABI Prism 7000 or 7900 Sequence Detection System, using SDS 1.1 software for allele discrimination (Applied Biosystems).

HLA-DQ typing was performed in the respective laboratories as previously described^[10,11].

Statistical analysis

Allelic and genotypic frequencies were compared by means of χ^2 tests or Fisher's exact test when necessary (expected values below 5). Statistical analyses were performed with the statistical package EpiInfo v5.00 (CDC, Atlanta, USA). Transmission disequilibrium test (TDT), which assesses the preferential transmission of one allele over the other from heterozygous parents to affected sibs, was used to analyze the familial data.

RESULTS

Allelic and genotypic frequencies for *CD209* in celiac disease patients and controls are shown in Table 1 for the first cohort studied (Hospital Virgen de las Nieves, Granada). No significant differences were observed in any case. A similar result was obtained when family data were analyzed (27 G alleles transmitted *vs* 33 not transmitted, $P = 0.26$). However, due to the overwhelming and potentially masking influence of HLA-DQ2 (DQA1*0501 and DQB1*02) on celiac disease, we divided the patients into two groups according to the presence (+) or absence (-) of those alleles. No allele of *CD209* was significantly increased in any group of patients ($P = 0.11$) (Table 2). However, the different genotype distribution was significant in both groups of patients. The small sample

Table 2 Genotypic frequencies in HLA-DQ2 (+) and HLA-DQ2 (-) patients from the Granada area

Genotype	DQ2 (-) patients (n = 7)	DQ2 (+) patients (n = 96)
AA	2	59
AG	5	31
GG	0	6

Table 3 Genotypic CD209 frequencies in DQ2 (-) (n = 29) and DQ2 (+) (n = 357) celiac patients and controls (n = 419) from the Madrid area

Genotype	DQ2 (-) patients n (%)	DQ2 (+) patients n (%)	Controls n (%)
AA	18 (62)	207 (58)	255 (61)
AG	6 (21)	131 (37)	142 (34)
GG	5 (17)	19 (5)	22 (5)

3*2 contingency table DQ2- vs DQ2+: $P = 0.017$; 3*2 contingency table DQ2- vs controls: $P = 0.019$; carriers A vs GG DQ2 (-) vs DQ2 (+): $P = 0.026$ OR = 3.71 (1.10-11.78); carriers A vs GG DQ2 (-) vs controls: $P = 0.023$ OR = 3.76 (1.14-11.73).

size of the DQ2 (-) group ($n = 7$) could be responsible for the lack of statistical significance, therefore we decided to repeat the study using a higher number of patients.

A second cohort was then studied (Madrid) (Table 3) and we did obtain significant results. Genotypic frequencies were significantly different when DQ2 (+) celiac disease patients were compared with DQ2 (-) patients ($P = 0.017$) or with controls ($P = 0.019$) and an even higher significance was observed when DQ2 (+) celiac disease patients and controls (grouped because there are no significant differences between them) were considered together ($P = 0.013$). The differences seemed to be mostly due to the increased susceptibility to celiac disease occurrence in DQ2 (-) individuals carrying two copies of the allele *CD209* -336G (AA + AG individuals vs GG individuals, $P = 0.021$, OR = 3.73 (1.18-11.03), DQ2 (-) vs DQ2 (+) individuals and controls considered together). A family study with samples from Madrid was also performed to determine the influence of *CD209* on celiac disease after DQ2 stratification. However, the scarce number of families with DQ2 (-) children did not allow us to obtain any conclusion (there were only 4 families and one of the parents was heterozygous for the studied polymorphism and the allele A was transmitted just in one case).

DISCUSSION

The contribution of HLA class II genes to CD susceptibility is for the moment in the most reproducible and well established fact regarding CD genetics^[12]. Thus, 92%-93% of celiac disease patients carry DQA1*0501-DQB1*02 (DQ2), in sharp contrast with only 28% of the Spanish healthy controls. However, less is known regarding

the genetic factors contributing to CD predisposition in patients lacking the DQ2 allele. In this study we analyzed for the first time the contribution of -336 A/G *CD209* genetic variant to CD susceptibility, investigating the contribution of this polymorphism in both DQ2+ and DQ2- CD patients. Interestingly, we have shown that the *CD209*-336 polymorphism seems to be a genetic risk factor for CD in DQ2- patients in our population. We observed that in DQ2- patients the *CD209*-336 GG genotype was significantly overrepresented, suggesting that additional predisposition factors are more relevant in patients lacking the major susceptibility determinant, namely the antigen presenting HLA class II molecule DQA1*0501-DQB1*02. According to the results obtained in this study, *CD209*, more precisely, the promoter allele-336G, is one of those additional secondary genetic factors.

The *CD209* -336G allele is also associated with increased predisposition to parenteral HIV infection^[13]. The G allele favors the binding to the ubiquitous transcription factor Sp1, but paradoxically the transcription rate from the G allele seems to be lower than that from the A allele^[8]. Perhaps the lower amount of DC-SIGN protein on the cell surface might reduce the surveillance activity of sentinel cells, and therefore may promote the persistence of pathogens in the gut. The continued presence of pathogens may underlie in turn the inflammatory down-regulation in celiac disease patients. In fact, it has been suggested that some bacterial infections might play a relevant role in CD development^[1,5] and the hypothesis proposed could support the maintenance or facilitation of bacterial infection in CD onset.

In summary, the *CD209*-336 polymorphism for CD susceptibility exists in DQ2- patients in our population. It is necessary to analyze the possible contribution of other C-type lectin receptors (mannose receptor, endo-180, SIGNR1, dectin-1, dectin-2) that are able to bind to exogenous ligands as interesting candidate genes in CD predisposition, especially in DQ2 negative patients.

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Expression, purification and bioactivity of human augments of liver regeneration

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Abstract

AIM: To construct the expression vectors for prokaryotic and eukaryotic human augments of liver regeneration (hALR) and to study their biological activity.

METHODS: hALRcDNA clone was obtained from plasmid pGEM-T-hALR, and cDNA was subcloned into the prokaryotic expression vector pGEX-4T-2. The recombinant vector and pGEX-4T-2hALR were identified by enzyme digestion and DNA sequencing and transformed into *E coli* JM109. The positively selected clone was induced by the expression of GST-hALR fusion protein with IPTG, then the fusion protein was purified by glutathione s-transferase (GST) sepharose 4B affinity chromatography, cleaved by thrombin and the hALR monomer was obtained and detected by measuring H thymidine incorporation.

RESULTS: The product of PCR from plasmid pGEM-T-hALR was examined by 1.5% sepharose electrophoresis. The specific strap was coincident with the theoretical one. The sequence was accurate and pGEX-4T-hALP digested by enzymes was coincident with the theoretical one. The sequence was accurate and the fragment was inserted in the positive direction. The recombinant vector was transformed into *E coli* JM109. SDS-PAGE proved that the induced expressive fusion protein showed a single band with a molecular weight of 41 kDa. The product was purified and cleaved. The molecular weights of GST and hALR were 26 kDa, 15 kDa respectively. The recombinant fusion protein accounted for 31% of the total soluble protein of bacterial lysate. hALR added to the culture medium of adult rat hepatocytes in primary culture and HepG₂ cell line could significantly enhance the rate of DNA synthesis compared to the relevant

control groups ($P < 0.01$).

CONCLUSION: Purified hALR has the ability to stimulate DNA synthesis of adult rat hepatocytes in primary culture and HepG₂ cells *in vitro*, and can provide evidence for its clinical application.

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Key words: Human augments of liver regeneration; Gene recombination; Expression; Purification; Fusion protein; Transformation; Biological activity

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INTRODUCTION

Transplantation of hepatocytes can be used to treat acute and chronic hepatic failure, and related hepatic diseases^[1,2]. Due to the difficulty to get satisfactory proliferation of transplanted cells, the hepatic function and superseding effect of the damaged hepatic parenchyma are not good^[3,4]. A large number of living hepatocytes are needed in the substituting period for metabolic disorder^[5]. But the source of donor hepatocytes is limited. All of above-mentioned factors influence hepatic transplantation.

Human augments of liver regeneration (hALR) is a stimulator of hepatic proliferation found in recent years. Compared to other growth factors, it can be used to restrain the growth of some tumor cells^[6,7]. Meanwhile, it can resist hepatic damage and reverse hepatic fibrosis^[8,9]. Application of this factor will improve the proliferating ability of culture *in vitro* and hepatocyte transplantation *in vivo*^[10].

MATERIALS AND METHODS

Materials

Plasmid pGEX-7-hALR containing 380 bp human regeneration promoter cDNA, prokaryote expression vector pGEX-4T-2 and host bacteria JM109 were conserved in our laboratory. Ligase T4 DNA, restrictive endonucleases *Bam*H1 and *Eco*R1, calf intestinal alkaline

phosphatase (CIP) and thrombin were obtained from American MBI Company. TaqDNA polymer dNTP was purchased from Promega Company. Standard proteins with a low molecular weight and calibration kit for SDS electrophoresis were purchased from Pharmacia Company MBI QIA-GEN gel callback sample box and emitting isotope H-TDR were bought from Shanghai Shenggong. Wistar male rats were provided by the Animal Center of our school.

Methods

In this research, recombinant hALRcDNA was cloned into plasmid pGEX-4T-2 with genes of fusion protein, and then recombinant vector was transformed into *E coli* JM109. After digestion and sequencing, fusion protein expression was detected by SDS-PAGE electrophoresis. Affinity chromatography was used to purify GST-hALR after digestion by thrombin. After hALR was cloned into an expression plasmid of eukaryon with expressive green fluorescence protein (EGFP) as reporter gene, LO₂ hepatocytes were transfected by liposome transfection method. Transfection efficiency and instantaneous expression were observed after recombination. The biological activity of hALR was studied by detecting DNA synthetic rate as previously described^[11].

Oligonucleotide primer: According to the sequence of ALR, the specific primer of hALRcDNA was designed and the sequence identified by enzymes *Bam*HI and *Eco*RI was used to observe and reverse the primer. The upper primer p1 was 5'-CGGGATATGGGGACGCAGCAGAAGAAGCGGGAC-3', the lower primer p2 was 5'-GCCAATTCGGCTAGTCACAGGAGCCGACCTTCC-3'. After 35 cycles of amplification at 95°C for 1 min, at 64°C for 1min, at 72°C for 1min, 1.5% agarose gel electrophoresis was used to identify the purified genes.

DNA recombination and identification: Gene expression sequence of hALRcDNA was cloned into pGEX-4T-2 vector, and identified with enzymes *Bam*HI and *Eco*RI. The results were proved by Shanghai Physiologic Center.

Expression and purification of recombinant gene induced by eukaryon: Recombinant positive clone containing pGEX-4T-2-hALR was put into LB culture medium at 37°C, and IPTG (final concentration was 1 mmol/L) was added to induce expression for 4 h, then the bacilli were collected and broken by ultrasound pulverizer (4°C, 12 000 r/min, 20 min). The supernatant was collected and put into 2 × loading buffer. The expression of recombinant fusion protein was identified by SDS-PAGE electrophoresis.

Recombinant plasmid transfection of LO₂ hepatocytes: LO₂ hepatocytes were transferred into a cell culture flask in DMEM containing penicillin 100 U/mm, streptomycin 100 U/mm, 10% calf serum, tri-distilled water and incubated in 5% CO₂ at 37°C.

One day before liposome transfection, LO₂ hepatocytes 4 × 10⁴ were loaded onto coverslips, put into 6-well plates and cultured with 1.5 mL DMEM containing 10% calf serum 5% CO₂ at 37°C until cells grew to 80% coverage. The cells were washed 2 times with normal saline. DNA-liposome complex was put into two six-well plates, 0.5 mL

of DMEM-SF without antibiotics was added into each well and the plates were slightly shaken to make the DNA-liposome mixture flow evenly over the cell surface. Cells were cultured in 5% CO₂ at 37°C for 5 h without change of supernatant, 0.5 mL of 30% of calf serum was added into DMEM and cultured for 24 h, then the medium was washed two times with normal saline, 1.5 mL of 10% of serum was added into DMEM.

Instantaneous expression of recombinant plasmid in LO₂ hepatocytes: LO₂ hepatocytes were cultured in different groups. The first group was vaccinated on coverslips after digestion by pancreatin, put into six-well plates and incubated. The cells were observed under fluorescent microscope after 12, 24, 48 h of transfection. The second group of hepatocytes was transfected with LO₂ hepatocytes of recombinant plasmid in DMEM-SE medium. The third group was transfected with blank material into blank plasmid, the upper clear liquid was drawn after one week of culture, then 30% of volume was added into routinely cultured HepG₂ cancerous cell medium and vaccinated into 12-well plates 5 × 10⁵/mL, the HepG₂ cells were incubated in routine culture medium in control group. Luciferase was added into each well after cultured for 24 h. The cells were collected, washed repeatedly with PBS, the infiltrative membrane was dried by airing, and put into a scintillation bottle with scintillation liquid. DNA synthesis rate (CPM value) was detected by a scintillometer. The third group was cultured routinely for 72 h, G418 200 µg/mL of calf serum was added to screen cells. The liquid was changed every 3 d.

Detection of biological activity of expression hALR: The primary hepatocytes were isolated by hepatic perfusion. In brief, 2.5 mL of mixed hepatocyte suspension and 15 mL of Percoll were centrifuged at 88 r/min, for 13 min, then the cells were collected and counted using a optical microscope. The separated hepatocytes and HepG₂ tumor cells were incubated respectively in cell culture plates, and then the supernatant was discarded. The third trial group was established by adding DMEM liquid separately containing hALR 5 ng/mL, 10 ng/mL, and 20 ng/mL.

Statistical Analysis: The data were dealt with by double factor variance analysis with SPSS 10.0 software package.

RESULTS

Construction and identification of pGEX-4T-2hALR: The reaction result of PCR augmentation of hALRcDNA (Figure 1) was coincident with hALRcDNA. There were the endonuclease sites of *Bam*HI, *Eco*RI on 5' - 3' ends.

Construction of pGEX-4T-2hALR after hALRcDNA acquired by PCR augmentation was performed using restriction endonucleases *Bam*HI and *Eco*RI. Gene segment was recycled by 1.5% sepharose electrophoresis. pGEX-4T-2 was cleaved by *Bam*HI and *Eco*RI, then the gene segment was recycled. Transformed susceptible bacteria JM109 were screened using ampicillin to yield 12 monoclonal colonies. The recycled vector segment was not recombined and cultured directly in the contrast LB culture medium after phosphorylation, but no clone appeared. Five monoclonal colonies were selected

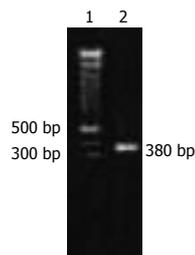


Figure 1 PCR 1.5% Gel electrophoresis. Lane1: 100 bp Ladder Marker; Lane2: hAL-RcDNA.

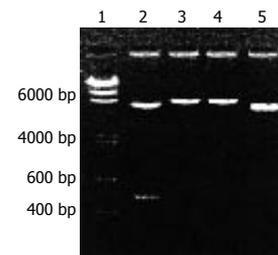


Figure 2 pGEX-4T-2-hALR digestion analysis. Lane1: λ DNA/*Hind*III marker; Lane2: pGEX-4T-2-hALR/B, E; Lane3: pGEX-4T-2-hALR/*Bam*HI; Lane4: pGEX-4T-2-hALR/*Eco*RI; Lane5: pGEX-4T-2-B, E. (B: *Bam*HI, E: *Eco*RI).

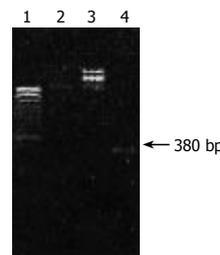


Figure 4 Comparison of inclusion body and supernatant. Lane1: 100 bp Ladder marker; Lane2: inclusion body; Lane3: supernatant.

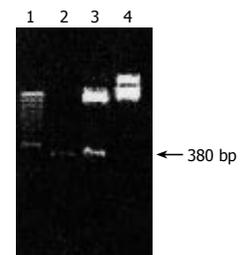


Figure 5 Digestion pEGFP-C1 and hALR. Lane1: 100 bp Ladder Marker; Lane2: pEGFP-c1 E/B; Lane3: pEGFP-c1; Lane4: hALR E/B.

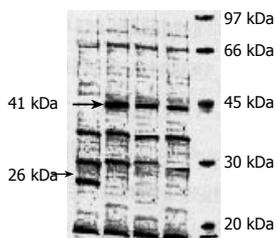


Figure 3 Expression of GST-hALR fusion protein. Lane1: marker; Lane2: induced 1h; Lane3: induced 3h; Lane4: induced 8h; Lane5: control.

randomly. The plasmid was digested by *Bam*HI and *Eco*RI to yield a band 5.3 kb (Figure 2). This indicated exogenous DNA was inserted. After digestion by *Bam*HI and *Eco*RI and recombinant plasmid, two bands of 4.9 kb and 380 bp were produced. In contrast to non-vector double cutting enzyme, 380 bp external DNA was inserted in pGEX-4T-2 between the cutting site of *Bam*HI and *Eco*RI. The recombinant plasmid-inserted 380 bp hALR was acquired. The structure of plasmid was completely satisfactory. Five positive clones were acquired. Therefore, a complete hRcDNA segment was inserted into pGEX-4T-2 plasmid between *Bam*HI and *Eco*RI.

Expression of hALR in *E. coli*: Expression of fusion protein GST-hALR was induced after the bacteria containing recombinant expression plasmid pGEX-4T-2hALR were induced by IPTG. The split product was detected by SDS-PAGE electrophoresis (Figure 3), a new band of 41 kDa was found in the recombinant bacteria, while the nonfusion protein GST band expressed by pGEX-4T-2hALR nonvector transformed bacteria was 26 kDa, indicating that molecular weight of the fusion protein was 41 kDa, the part of GST was 26 kDa, and the molecular weight of hALR was 15 kDa.

After the recombinant bacteria were split by ultrasound, its inclusion body and supernatant were detected by SDS-PAGE electrophoresis (Figure 4). The significant band of fusion protein (GST-hALR) was seen at the site of protein 41 kDa in upper clean liquid, but the weak band was seen at the same site in inclusion body, indicating that the fusion protein was expressed mainly in the soluble form in the upper liquid.

To identify the best expression condition, the expression period was analyzed. The result showed that the expression product of fusion protein in the LB liquid culture medium was ascended in 0-8 h after the

transformed bacteria were induced with IPTG under 30°C. By the gel scanning system, the expression amount of GST-hALR at 8 h covered 31% soluble protein of bacteria.

Fusion protein in the supernatant was analyzed by SDS-PAGE, then deoxidized glutathione S-transferase(GST) was used to purify GST-hALR. The vector of pGEX-4T-2 had the cutting site of the thrombin between GST and multicloning, the function of thrombin could separate GST and hALR in the fusion protein. After fusion protein was split by thrombin, split GST and undigested fusion protein were filtered by glutathione sepharose 4B affinity chromatography. The purified hALR was acquired, and there were also single clean strips in the map of SDS-PAGE. Molecular weight was about 15 kDa. The concentration of the purified hALR was about 90.5% and the hALR protein concentration was about 0.87 mg/mL.

Construction and identification of recombinant plasmid pEGFP-C1-hALR: In the map of pEGFP-C1 multiple clones, *Eco*RI and *Bam*I II were in the region of MCS. *Eco*RI was in the front, *Bam*I II was in the rear, indicating that hALR could be inserted positively. pEGFP-C1 plasmid and ALRcDNA segment were cut respectively by *Eco*RI and *Bam*I II. The site of cut hALR PCR segment was at 400 bp, two light bands were seen in the uncut pEGFP-C1 plasmid due to the difficult spatial conformation of cycled plasmid. pEGFP-C1 was cut to linear molecule by enzymes, a light band was seen by electrophoresis, the velocity rate was slightly faster than that of uncut pEGFP-C1 plasmid by enzymes (Figure 5).

When the kanamycin plate was incubated overnight at 37°C, 15 clones sprouted which were cultured overnight with kanamycin LB culture medium at 37°C.

The site of PCR augmentation band of recombinant plasmid was similar to that of PCR augmentation band of bank DNA. There were two characteristic bands after recombinant plasmid was cut by enzymes. One band was at 400 bp, the other band was at 4.7 kb, suggesting that hALRcDNA was cloned into pEGFP-C1. Recombinant plasmid pEGFP-C1-hALR was constructed successfully.

The result of PCR was coincident with the theoretical one, the sequence of recombinant plasmid pEGFP-C1-hALR was right.

OD value of pEGFP-C1-hALR detected by ultraviolet spectrophotometer is shown in Table 1.

pEGFP-C1-hALR and its sequence might be used to transfect cells and to observe the expression condition of

Table 1 OD value of pEGFP-C1-hALR detected by ultraviolet spectrophotometer

Sample	abs 280.0 nm	abs 260.60	bkgabs 320.0 nm	260.0 nm	280.0 nm	Protein $\mu\text{g/mL}$	Nucleic acid $\mu\text{g/mL}$
1	0.0319	0.0205	0.0057	1.7679	0.5657	3.1539	1.1124
	0.0337	0.0234	0.0086	1.6953	0.5899	3.9632	1.0440
2	0.0152	0.0073	-0.0009	1.9486	0.5132	0.6326	0.7177
	0.0159	0.0101	-0.0009	1.8485	0.5410	1.6853	0.8892



Figure 6 Expression of green fluorescence protein of recombinant pEGFP-C1-hALR in LO₂ hepatocyte.

green fluorescence protein.

Expression of recombinant plasmid pEGFP-C1-hALR in LO₂ hepatocytes: LO₂ hepatocytes with recombinant plasmid pEGFP-C1-hALR for 12 h. One coverslip was taken from the 6-well plates transfected and observed under microscope. Cells were attached to the wall, cleavage phase of some cells were seen. The medium was washed 2 times with normal saline, digested by pancreatin. The cells were counted. Another coverslip was taken from the plates to observe the expression of green fluorescence under fluorescence microscope (Figure 6).

The green fluorescence was expressed in some cells after transfection of liposome for 12 h. Most of the expression was weak.

Detection of biological activity of hALR: The hepatocytes were separated by hepatic perfusion with IV collagenase. After separation and purification, the number of cells was about $2 \times 10^8/\text{mL}$, the survival rate of cells was about 94%. CPM value detected by ³H-thymidine incorporation method was used to observe the influence of DNA synthesis rate of hALR on primary cultures of hepatocytes and HepG₂ cells cultured *in vitro* (Tables 2 and 3).

Seen from the above table, recombinant hALR might stimulate DNA synthesis of primary hepatocytes and HepG₂ cells compared to control group. There was a significantly statistical difference ($P < 0.01$). The different dose of hALR had different effects on primary hepatocytes and HepG₂ cells. Comparison of mean value between each group, there was a significant statistical difference ($P < 0.01$), indicating that DNA synthesis rate increased gradually with increasing dose of hALR.

Table 2 Influence of different concentrations of hALR on DNA synthesis of primary rat hepatocyte CPM (mean \pm SD)

CPM	1 Without hALR control group $n = 6$	2 5 ng/mL hALR medium group $n = 6$	3 10 ng/mL hALR medium group $n = 6$	4 20 ng/mL hALR medium group $n = 6$
1	1125 \pm 158	$P < 0.01$	$P < 0.01$	$P < 0.01$
2	1802 \pm 127		$P < 0.01$	$P < 0.01$
3	2764 \pm 445			$P < 0.01$
4	3344 \pm 417			

Table 3 Influence of different concentrations of hALR on DNA synthesis of HepG₂ cell CPM (mean \pm SD)

CPM	1 Without hALR control group $n = 6$	2 5 ng/mL hALR medium group $n = 6$	3 10 ng/mL hALR medium group $n = 6$	4 20 ng/mL hALR medium group $n = 6$
1	1226 \pm 158	$P < 0.01$	$P < 0.01$	$P < 0.01$
2	1802 \pm 127		$P < 0.01$	$P < 0.01$
3	2764 \pm 445		$P < 0.01$	
4	3344 \pm 417			

DISCUSSION

Due to limited donor source and complication of the operation itself, liver transplantation *in situ* in treatment of terminal end hepatic disease has been widely used. The technique of hepatocyte transplantation developed at the end of the 1970's has gained more and more attention because one donor can provide hepatocytes for several recipients and the transplant technique is very simple^[8,12].

We studied the biological activity of the expression product by constructing expression vector of pronuclei and eukaryons of human augments of liver regeneration in order to provide experimental evidence for the study of hepatocellular source of hALR hepatocyte transplantation modified by gene and the clinical application of hALR. But there are three major problems in the transplantation of hepatocytes, namely the transplanting location, immune reaction, and proliferation of hepatocytes^[8]. When transplanted in the parenchyma of spleen or in the abdominal cavity, hepatocyte transplantation always has better effects^[13]. When biological materials such as biological compatibility membrane are used, hollow fibers may form a barrier for the transplanted hepatocytes, which has solved the problem of host immune repulsive action, and increased interest in xenogenic hepatocyte transplantation^[11,14]. Transplanted hepatocytes perform the temporary maintenance of hepatic function and the temporary substitution for the damaged hepatocytes by proliferation *in vivo*. Since the desired results of culture *in vitro* and proliferation *in vivo* are difficult to get, the final result is not so good^[3,4]. Furthermore, to treat congenital metabolic defects with hepatocyte transplantation, a large quantity of hepatocytes that can express exogenic genes are needed^[15]. The traditional methods to improve the transforming rate such as 70% resection of liver, damaging the liver with CCL₄, can bring damage to the liver of the recipient. Therefore how to acquire a stronger

proliferation ability, how to culture plenty of cells *in vitro*, while ensure their biological activity and long lifespan are crucial problems.

With the advance in modern gene engineering techniques, especially combination of gene engineering techniques with modern surgical transplantation technique, a new curative technique is produced, namely genetically-modified cell transplantation, proliferation of the transplanted hepatocytes is possible to be solved through the modification of hepatocytes. HALR is a stimulator of hepatocyte proliferation that can bear temperature, with specificity for liver but without species specificity. HALR can accelerate synthesis of DNA of primary generative hepatocytes cultured *in vitro* and hepatogenous hepatic tumor cells and the proliferation of the cells, while it has no influence on the nonhepatic cells, showing that it has a specific role in accelerating proliferation of the hepatocytes. HALR is an important regeneration-regulating factor because it can accelerate the hepatocyte proliferation. HALR may accelerate proliferation of hepatic cells in 1/3 of models with partial resection of liver, indicating it is an important regeneration-regulating factor^[11,16-18]. In addition, it can protect the liver against damage, reverse hepatic fibrosis^[19,20] and take part in cell immune action. All these suggest that proliferation of the transplanted hepatocytes can be suppressed by the use of enhanced and modified regenerative cells. Regeneration-enhanced factor as a kind of special stimulating factor of growth of hepatic cells, has gained more and more attention of domestic and foreign scholars. It has a prospect to be used as an effective medicine to treat severe hepatic diseases.

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RAPID COMMUNICATION

Isolated antibody to hepatitis B core antigen in patients with chronic hepatitis C virus infection

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Abstract

AIM: To evaluate the prevalence of isolated anti-HBc in patients with chronic hepatitis C virus (HCV) infection, and its relation to disease severity.

METHODS: We screened all patients with chronic HCV infection referred to King Faisal Specialist Hospital and Research Center for hepatitis B surface antigen (HBsAg), antibody to hepatitis B surface antigen (anti-HBs), and anti-HBc. One hundred and sixty nine patients who tested negative for both HBsAg and anti-HBs were included in this study.

RESULTS: Pathologically, 59 had biopsy-proven cirrhosis and 110 had chronic active hepatitis (CAH). Of these 169 patients, 85 (50.3%) tested positive for anti-HBc. Patients with CAH had significantly higher prevalence of isolated anti-HBc than patients with cirrhosis, 71 (64.5%) and 14 (23.7%) respectively ($P < 0.001$). Twenty-five patients were tested for HBV DNA by qualitative PCR. The test was positive in 3 of them (12%; occult HBV infection).

CONCLUSION: Isolated anti-HBc alone is common in Saudi patients with chronic HCV infection, and is significantly more common in those with CAH than those with cirrhosis. Therefore, a screening strategy that only tests for HBsAg and anti-HBs in these patients will miss a large number of individuals with isolated anti-HBc, who may be potentially infectious.

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Key words: Cirrhosis; Chronic hepatitis; Dual infection; Co-infection; Hepatitis B screening; Super-infection

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INTRODUCTION

Hepatitis B virus (HBV) and hepatitis C virus (HCV) infections account for a substantial proportion of acute and chronic liver diseases world-wide^[1-4], including Saudi Arabia^[5-7]. Both viruses share similar risk factors and modes of transmission and as a consequence, combined HBV and HCV infection is frequent especially in areas endemic for HBV, and among people at high risk of parenteral infection^[1-5]. Indeed, patients with HCV-related chronic liver disease (CLD) frequently show markers of previous HBV infection. Moreover, they may carry occult HBV infection. These features might influence clinical and biochemical features as well as stage of disease.

Screening patients with chronic HCV for the coexistence of HBV infection is usually based on serum testing for the presence hepatitis B surface antigen (HBsAg). This screening is critical, because HBV infection needs treatment if present, can be prevented by vaccination if absent, and may adversely affect liver pathology and the response to therapy.

Isolated hepatitis B core antibody (anti-HBc) represents either resolved HBV infection with loss of hepatitis B surface antibody (anti-HBs); occult chronic HBV infection with levels of the HBsAg below the limits of detection; or a false-positive test result. Many western publications have reported that 2%-5% of healthy blood donors have isolated anti-HBc^[8-10]. Similarly, isolated anti-HBc was reported in 125 (2%) of 6035 consecutive Saudi blood donors^[11]. In addition, we have previously shown that the prevalence of isolated anti-HBc in HBsAg negative potential liver donors is 32%, and is significantly higher in non-Saudi nationals (41.3%) compared with Saudi citizens (16.7%)^[12]. However, the prevalence of isolated anti-HBc in patients with chronic HCV infection has not been studied. Therefore, the aims of the present study were to

evaluate the prevalence of “isolated anti-HBc” in patients with chronic HCV infection, and to assess its relation to disease severity.

MATERIALS AND METHODS

The subjects included in this study are referrals to King Faisal Specialist Hospital and Research Center (KFSH&RC), Riyadh, Saudi Arabia, between January and December 2004.

Subjects included in this study ($n = 169$) are patients with confirmed chronic HCV infection genotype 4, and negative HBsAg and anti-HBs. HCV antibody testing was performed with an enzyme immunoassay (Abbott HCV EIA 2.0; Abbott Laboratories) and positive polymerase chain reaction (PCR) for HCV RNA for more than 6 mo. Routine liver function tests and diagnostic liver biopsy were done to all patients. Histological diagnosis was evaluated, using the modified Knodell's histological activity index,^[13] by a pathologist who was unaware of the patient's serological data.

Tests for HBsAg, anti-HBs, and anti-HBc were done using (Auszyme; Abbott Laboratories), (Ausab EIA; Abbott Laboratories) and (Hepatitis B Virus Core Antigen Corzyme; Abbott Laboratories) respectively. In addition, sera from a subgroup of patients ($n = 25$) who tested positive ($n = 19$) and negative ($n = 6$) for anti-HBc only were further tested for the presence of HBV DNA using nested-PCR with primers deduced from the core region. In the cases tested positive ($n = 3$), viral load was performed using HBV-Amplicor Monitor (Roche Molecular System). The lower limit of detection for this assay is 2000 copies/mL. As this study concentrates on isolated anti-HBc in patients with chronic HCV infection, subjects with overt HBV infection alone or in combination with HCV were excluded.

This study was approved by the Institutional Research Committee and the Review Board of the KFSH&RC.

Statistical analysis

Results were collected in a Microsoft *Excel* spread sheet, and expressed as mean \pm SD unless otherwise stated. Data were examined by the Pearson's correlation coefficient and two-tailed paired, un-paired Student's *t*-tests, Chi-Square, as appropriate Using SPSS Statistical Package version 10 (SPSS Chicago, IL, USA). Statistical significance was taken at the 5% level.

RESULTS

A summary of the demographic patient characteristics is shown in Table 1. A total of 169 patients with chronic HCV infection were included. Based on liver function testing, positive serum anti-HCV, positive HCV RNA, and liver histopathology, 59 patients had biopsy-proven cirrhosis and 110 had CAH. Of these 169 patients, 85 (50.3%) tested positive for anti-HBc. The potential mode of transmission was identified in 123 patients (72.8%), and includes blood transfusion, surgical and dental interventions before the availability of HCV testing. The remaining cases may fall under intra-familial spread. We

Table 1 Subjects characteristics ($n = 169$)

Variable	Mean	SD
Age (yr)	44.0	18.0
Sex		
Female	57 (33.7%)	
Male	112 (66.3%)	
INR	1.1	0.26
AST (U/L)	81.1	97.3
ALT (U/L)	74.4	88.3
GGT (U/L)	117.8	194.9
Bilirubin (μ mol/L)	29.9	53.1
ALP (U/L)	149.9	111.3
Albumin (g/L)	35.7	6.2
Urea (mmol/L))	6.9	6.4
Creatinine (μ mol/L)	144.5	196.6
Liver histopathology		
Cirrhosis	59 (34.9%)	
CAH	110 (65.1%)	
Anti-HBc		
+ve	85 (50.3%)	
-ve	84 (49.7%)	

Data are expressed as mean \pm SD or number (%). NS = not significant. ALP; alkaline phosphatase. ALT; alanine aminotransferase. AST; aspartate aminotransferase. CAH; chronic active hepatitis. GGT; Gamma-glutamyl transferase. HBcAb; hepatitis B core antibody. INR; international normalization ratio.

did not test the partners for HCV or HBV infection in this study. Therefore, the sexual transmission can not be excluded.

Comparison between patients with positive anti-HBc and negative anti-HBc

Compared with patients with negative anti-HBc, patients with chronic HCV infection and positive isolated anti-HBc antibody had significantly higher mean age, and significantly lower mean ALT levels ($P < 0.001$ and $P < 0.5$ respectively; Table 2). The mean AST levels were also less in patients with negative HBc antibody, though it did not reach statistical significance. In addition, and despite their higher mean age, patients with isolated anti-HBc antibody pathologically showed a significantly less cirrhotic and significantly more chronic hepatitis picture ($P < 0.001$, Table 2).

HBV DNA Polymerase Chain Reaction

Qualitative PCR testing for HBV DNA was performed in 25 cases (14.8%); 6 were negative for anti-HBc, and 19 were positive for isolated anti-HBc antibody. Positive serum HBV DNA was detected in 3 patients, all had positive isolated anti-HBc (15.8% of the 19 cases with positive anti-HBc, and 12% of the 25 cases tested by PCR), two were cirrhotic and the third was in the chronic inflammatory stage. The viral load in these 3 patients was 4820, 3800, and 4300 copies/mL respectively. Therefore, they represent an “occult HBV Infection”, and are potentially infectious.

Independent predictors of cirrhosis

Multivariate regression analysis of variables of interest (age, sex, ALP, AST, ALT, urea, creatinine, International

Table 2 Comparison between patients with positive anti-HBc and those with negative anti-HBc

Variable	Patients with HCV & positive anti-HBc (n = 85)	Patients with HCV & negative anti-HBc (n = 84)	P value
Age (yr)	39.1 ± 18.3	48.9 ± 16.3	< 0.001
Sex			
Female	33 (38.8)	24 (28.6)	NS
Male	52 (61.2)	60 (71.4)	
INR	1.1 ± 0.3	1.1 ± 0.2	NS
AST (U/L)	68.1 ± 44.5	94.4 ± 130.1	NS
ALT (U/L)	61.0 ± 39.9	88.8 ± 119.0	< 0.05
GGT (U/L)	100.2 ± 134.0	137.2 ± 245.8	NS
Bilirubin (μmol/L)	30.0 ± 50.1	29.7 ± 56.6	NS
ALP (U/L)	133.2 ± 89.5	168.0 ± 129.1	NS
Albumin (g/L)	35.9 ± 6.1	35.4 ± 6.3	NS
Urea (mmol/L)	6.2 ± 4.3	7.8 ± 8.0	NS
Creatinine (μmol/L)	117.5 ± 138.9	1792.9 ± 240.6	NS
Liver pathology			
Cirrhosis	14 (16.5)	45 (83.5)	< 0.001
CAH	71 (53.6)	39 (46.4)	

Data are expressed as mean ± SD or number (%). NS = not significant; ALP: alkaline phosphatase; ALT: alanine aminotransferase; AST: aspartate aminotransferase; CAH: chronic active hepatitis; GGT: Gamma-glutamyl transferase; HBcAb: hepatitis B core antibody; INR: international normalization ratio.

Normalization Ratio, bilirubin, albumin, +ve anti-HBc) revealed the presence of raised INR ($P = 0.002$), increased ALP ($P = 0.006$), and -ve serum anti-HBc antibody ($P = 0.0001$) to independently predict liver cirrhosis in the studied cohort (Table 3).

DISCUSSION

The findings of this study suggest that > 50% of patients with chronic HCV infection for whom there is no serological evidence for HBV, when screened with HBsAg and anti-HBs, will be positive for anti-HBc antibody especially those in the chronic inflammatory stage of the disease.

HCV-infected patients should be tested for HBV markers to determine those who should receive HBV vaccination and those who need anti-HBV treatment. For patients with chronic HCV infection, prevention of HBV infection is critical, because this viral infection can be particularly severe and may adversely affect disease outcome.

Isolated Anti-HBc was previously reported to exist in 2% of 6035 Saudi blood donors^[11]. A figure that is similar to what has been reported elsewhere^[8-10]. The high prevalence of isolated anti-HBc antibody in patients with chronic HCV infection detected in this study (50.3%) has previously been reported in a similar study in patients with HCV with or without HIV-1 coinfection^[14]. This reflects the similar mode of transmission, high endemicity of both infections. Indeed, the detection of HBV DNA by PCR in these patients rules out the possibility of false positive results and confirms the diagnosis of occult coinfection.

Occult HBV infection may be manifested by having negative test results for HBsAg, but positive results for

Table 3 Multivariate regression analysis for the predictors of liver cirrhosis

Variable	B	P value	Exp (B)	95% CI for Exp (B)	
				Lower	Upper
Age	-0.01	NS	1.0	0.95	1.02
Sex	0.64	NS	1.9	0.55	6.45
INR	7.69	0.002	2182	18.01	264549
AST	-0.01	NS	1.00	0.99	1.00
Bilirubin	0.00	NS	1.00	0.98	1.01
ALP	-0.01	0.006	1.00	0.99	1.00
Albumin	-0.02	NS	0.98	0.98	1.11
Urea	0.05	NS	1.10	0.87	1.27
Creatinine	0.00	NS	1.00	1.00	1.00
+anti-HBc	-2.63	0.0001	0.07	0.02	0.28
Constant	-3.36	NS			

NS: not significant; CI: confidence interval; PCR: polymerase chain reaction; INR: international normalization ratio; ALP: alkaline phosphatase.

HBV DNA. The frequency of occult HBV infection in patients with anti-HBc is controversial. In one study, occult HBV infection was reported in 33% of subjects with chronic liver disease due to HCV infection and was more frequent in subjects with isolated anti-HBc^[15]. Diagnosing occult HBV infection requires sensitive HBV-DNA PCR assay. The postulated mechanisms underlying occult HBV infection include mutations of HBV-DNA sequence, formation of immune complexes-containing HBV, an integration of HBV-DNA into host's chromosomes, HBV infection of leucocytes, altered host immune responses, and interference by other viruses. The clinical implications of occult HBV infection involve different clinical aspects including first; harboring potential risk of HBV transmission through blood transfusion, hemodialysis, and organ transplantation; second, causing cryptogenic liver disease; third, contributing to the development of acute exacerbation or even hepatocellular carcinoma; and fourth, affecting disease progression and response to treatment of patients with chronic HCV infection^[16].

The precise prevalence of occult HBV infection is variable. In the present study, we found occult HBV infection in 15.8% in patients with chronic HCV infection with positive anti-HBc antibody. Higher figures were detected in other studies. For example, using PCR with primer pairs from three different regions of the HBV genome, Jilg *et al* demonstrated that 32.9% of patients with anti-HBc alone are positive for HBV DNA, the majority of them showing very low HBV concentrations^[17]. Also, Feraro *et al* 2003 found occult HBV infection in 7 out of 22 patients with HCV. However, this was unrelated to anti-HBc status^[18]. Similarly, occult HBV infection was detected in 42% of HIV-infected patients, and was significantly common in subjects with concomitant HCV infection (80%) than those with HIV-1 infection alone^[14]. This supports the suggested policy of donor exclusion based on the anti-HBc and anti-HBs serology as a means to eliminate low grade carriers of HBV in endemic areas without jeopardizing the blood supply. This variation in the prevalence of occult HBV may be related to differences in prevalence of each viral infection in

different communities, differences in the PCR technique, or population selection bias. Highly sensitive, quantitative, and functional molecular analyses of HBV, combined with a well-designed prospective clinical assessment will provide the best approach for the future study of occult HBV infection.

The higher prevalence of anti-HBc antibody in patients with chronic HCV hepatitis than in patients with cirrhosis observed in this study may be explained by a possible inhibitory effect exerted by HBV on HCV replication^[19]. In agreement with this, a study by Kao *et al* showed that occult HBV infection does not have clinical significance in patients with chronic hepatitis C residing in areas where HBV infection is endemic^[15]. Furthermore, occult HBV infection had no effect on early response to PEG-INF alpha^[20]. Further follow up into the maintenance and post-treatment phases will clarify if and when occult HBV affects the sustained viral response. However, Giannini *et al* have reported a different observation^[21]. In their study, the positivity for markers of HBV infection was more frequent in the cirrhotic group as compared to patients with CAH. They also reported higher histological grading and scoring in patients with CAH with anti-HBc^[21]. Reasons of this disagreement are not clear, but a difference in the HCV genotype and duration of infection may be implicated, as most of our patients are genotype 4, while those included in Giannini study were Genotype 1b^[21]. Other factors that may aggravate the histological scoring and grading in the study of Giannini *et al* such as alcohol intake, may have contributed to this difference.

Finally, subjects with isolated anti-HBc who test negative for HBV DNA may have cleared HBV infection and lost undetectable anti-HBs or have a false-positive test result for anti-HBc due to cross-reacting antibodies present in individuals with HCV infection. For patients with isolated anti-HBc who have cleared HBV infection and have undetectable levels of anti-HBs, immunization with HBV vaccine may result in an anamnestic response, which is the presence of detectable anti-HBs within a few weeks after the first vaccination^[22,23].

The possible limitations of the present study include; First, KFSH&RC is a tertiary center, which raises a question regarding the generalizability of the findings. However, studies conducted in Europe show similar results^[10,24,25], which suggest that our conclusions are not specific to a particular geographic region. Second, we tested subjects for total anti-HBc level, rather than IgG and IgM isotypes. Therefore, acute HBV infection, in which only IgM antibody is present, can not be differentiated from the chronic one. Finally, not all subjects who tested negative for HBsAg and anti-HBs and positive anti-HBc underwent testing for HBV DNA. However, the subgroup tested for HBV DNA revealed positive cases (3 in 19, 15.8%), confirming the conclusions of this study. Missing isolated anti-HBc positivity, which represents occult HBV infection in > 10% of cases may have serious implication if these cases were used in organ donation, blood transfusion. They may also be misdiagnosed as cryptogenic liver disease, in patients who test negative for anti-HBsAg and HCV antibody.

In conclusion, isolated anti-HBV sero-pattern is a

common finding in patients with chronic HCV infection, and may represent an occult infection in a smaller percent. A screening strategy that tests only for HBsAg and anti-HBs in HCV-infected patients will miss a large number of individuals with isolated anti-HBc, and a smaller number of patients with occult HBV infection. Addition of anti-HBc antibody screening is recommended, to avoid the adverse implications of missed HBV occult co-infection

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Endoscopic scoring of late gastrointestinal mucosal damage after adjuvant radiochemotherapy

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Abstract

AIM: To evaluate late effects of chemoradiation on gastrointestinal mucosa with an endoscopic scoring system and compare it to a clinical scoring system.

METHODS: Twenty-four patients going to receive chemoradiation after gastric surgery underwent endoscopy four wk after surgery and one year after the chemoradiation finished. Upper gastrointestinal findings were recorded according to a system proposed by World Organisation for Digestive Endoscopy (OMED) and clinical scoring was done with RTOG-EORTC radiation morbidity scoring systems.

RESULTS: There was no significant endoscopic difference in gastric and intestinal mucosa after chemoradiation ($P > 0.05$) and there was no association between endoscopic scores and clinical scores. Endoscopic changes were minimal.

CONCLUSION: Late effects after chemoradiation in operated patients with gastric cancers can be evaluated with an endoscopic scoring system objectively and this system is superior to clinical scoring systems.

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Key words: Radiochemotherapy; Gastric cancer; Endoscopy; Gastrointestinal mucositis

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INTRODUCTION

Irradiation of the gastric region has become popular in the setting of adjuvant treatment of resected high risk gastric cancer patients in recent years. It has been shown that adjuvant radiochemotherapy leads to a survival advantage in operated patients with gastric cancers^[1]. Gastrointestinal mucositis after radiation has been well studied especially in the oral cavity and oesophagus but chemotherapy induced gastrointestinal mucositis has been focused mainly on the small intestine^[2].

There is a lack of data about the mucositis of the stomach in the literature. Sartori *et al*^[3] reported gastric erosions after different chemotherapeutics but there is no study evaluating the effects of irradiation and chemotherapy on gastric and small intestinal mucosa and there is no standardised endoscopic scoring system to evaluate these effects. Ideally a mucositis scoring system should be objective, validated and reproducible across all clinical situations and applications. Because of the need of such a measurement instrument a number of different clinical scoring systems have been developed, most of them measuring oral mucositis (NCI-CTC, WHO, RTOG)^[4-6].

Endoscopy is considered to give the best estimation of gastrointestinal mucosal damage and is used extensively in the follow-up of operated gastrointestinal cancer patients. But there is no endoscopic evaluation or scoring system for chemoradiation induced gastrointestinal mucosal damage. Wachter *et al*^[7] proposed a scoring system for radiation induced proctitis based on the endoscopic terminology of the World Organisation for Digestive Endoscopy (OMED)^[8] and found it highly useful to show mucosal damage especially in asymptomatic patients (Table 1). Based on the study by Wachter *et al* we conducted the current research to evaluate the late effects of chemoradiation on gastrointestinal mucosa by using the terminology proposed by OMED.

MATERIALS AND METHODS

Patients

Between Jan 2001 and Jan 2005, a total of 102 patients received adjuvant chemoradiation for locally advanced

gastric adenocarcinoma (T3, T4 or nodal involvement) after operation at the Department of Radiation Oncology at Kartal State Hospital, Istanbul, Turkey. All these patients were invited to take part in the present study. Thirty-four of them accepted and gave informed consent.

Endoscopy was performed by the same experienced endoscopist at the Department of Gastroenterology in the presence of a resident who collected the data. It was done under standard conditions with a videogastroscope (Pentax 2980 type) 4 wk after the operation. Ten of 34 patients had total gastrectomy and esophagoenterostomy and were therefore excluded from the study. The remaining 24 comprised the study group and had a control endoscopy 1 year after the last day of radiation treatment.

In order to make a systematic description in patients who had a subtotal gastrectomy and any type of gastroenterostomy, the remaining stomach, stoma and 10-12 cm of intestine were examined endoscopically. The remaining stomach and the stoma was defined arbitrarily as part I representing the gastric mucosa and the intestinal tissue distal to the stoma as part II representing the intestinal mucosa.

Endoscopic findings were evaluated based on the terminology of OMED in both arbitrarily defined parts of examination separately.

Terminology of OMED as following:

-Telangiectasia:

- Grade 0: none
- Grade 1: single telangiectasia
- Grade 2: multiple non-confluent telangiectasia
- Grade 3: multiple confluent telangiectasia

-Congested mucosa:

- Grade 0: none
- Grade 1: focal reddening of the mucosa combined with an edematous mucosa
- Grade 2: diffuse not confluent reddening of the mucosa combined with an edematous mucosa
- Grade 3: diffuse confluent reddening of the mucosa combined with an edematous mucosa.

-Ulceration:

- Grade 0: none
- Grade 1: microulceration –superficial, < 1 cm²
- Grade 2: superficial > 1 cm²
- Grade 3: deep ulceration
- Grade 4: fistula, perforation

-Stricture:

- Grade 0: none
- Grade 1: > 2/3 of regular diameter
- Grade 2: 1/3 –2/3 of regular diameter
- Grade 3: < 1/3 regular diameter
- Grade 4: complete obstruction

-Necrosis:

- Grade 0: none
- Grade 1: necrosis

The highest grade of any one parameter qualified for the attribution to one of the given score levels regardless of the grade achieved in any other parameter. Pre and post treatment scores of the gastric and intestinal mucosa were compared.

Adjuvant treatment plan was similar to the intergroup study (INT- 0116) by MacDonald *et al*^[1]. Chemotherapy

Table 1 Endoscopy score system based on the system proposed by Wachter *et al*^[7]

	Congested mucosa	Telangiectasia	Ulceration	Stricture	Necrosis
Score 0	Grade 1	None	None	None	None
Score 1	Grade 2	Grade 1	None	None	None
Score 2	Grade 3	Grade 2	None	None	None
Score 3	Any	Grade 3	Grade 1	None	None
Score 4	Any	Any	Grade 2	Grade 1	None
Score 5	Any	Any	Grade > 3	Grade > 2	Any

Table 2 RTOG-EORTC late radiation morbidity scoring system (part for small/large intestine)

	Grade 0	Grade 1	Grade 2	Grade 3	Grade 4
Small/large intestine	None	Mild diarrhea, mild ramping, bowel movement 5 times daily, slight rectal discharge or bleeding	Moderate diarrhea and colic, bowel movement > 5 times daily, excessive rectal mucus or intermittent bleeding	Obstruction or bleeding requiring surgery	Necrosis, perforation, fistula

(CT) either with bolus fluorouracil and leucovorin or with infusional fluorouracil was administered before, together and after radiation. CT (bolus fluorouracil 425 mg/m² and leucovorin 20 mg/m² for 5 d or infusional fluorouracil 1 g/m² for 5 d) was initiated on day 1 and followed by radiochemotherapy (RT) beginning 28 days after the start of the initial cycle of CT. Radiochemotherapy consisted of 46 Gy at 2 Gy per d, 5 d per wk, for five wk either with bolus fluorouracil (400 mg/m²) and leucovorin (20 mg /m³) or infusional fluorouracil (1 g/m²) on the first 4 d and last 3 d of radiation. The radiation field included the tumor bed, and regional lymphatics with the technic described by Smalley *et al*. Tumor bed was defined by preoperative computed tomography or barium roentgenography. Perigastric, celiac, hepatoduodenal or hepatic portal and pancreaticoduodenal lymph nodes were included in the radiation field. Treatment was delivered either with 6 or with 15 MV photons by anterior and posterior parallel opposed fields. Kidneys were spared with personalised blocks. The equivalent of at least two thirds of one kidney was spared from the radiation field. One month after the completion of radiotherapy, two cycles of CT either with bolus fluorouracil (425 mg/m²) and leucovorin (20 mg/m²) or infusional fluorouracil (1 gr/m²) for five days were given one month apart.

For each patient side effects of radiation were documented by use of EORTC/RTOG score at the end of one year and endoscopic findings were compared with small/large intestine items of the morbidity scale (Table 2).

Statistical analysis

Statistical analysis was performed with Pearson Chi-square test. $P < 0.05$ was considered significant.

Table 3 Posttreatment endoscopic scores for gastric mucosa and their correlation with RTOG-EORTC scores

Post treatment endoscopic scoring for gastric mucosa	RTOG-EORTC late radiation morbidity scoring (small/large intestine)				
	0	1	2	3	4
0 (n = 13)	5	3	5	0	0
1 (n = 9)	5	0	4	0	0
2 (n = 0)	0	0	0	0	0
3 (n = 0)	0	0	0	0	0
4 (n = 2)	0	1	1	0	0
5 (n = 0)	0	0	0	0	0

Table 4 Posttreatment endoscopic scores for intestinal mucosa and their correlation with RTOG-EORTC scores

Post treatment endoscopic scoring for intestinal mucosa	RTOG-EORTC late radiation morbidity scoring (small/large intestine)				
	0	1	2	3	4
0 (n = 19)	9	3	7	0	0
1 (n = 4)	1	1	2	0	0
2 (n = 0)	0	0	0	0	0
3 (n = 0)	0	0	0	0	0
4 (n = 1)	0	0	1	0	0
5 (n = 0)	0	0	0	0	0

RESULTS

All of 24 patients (7 females and 17 males) completed the study and there were no dropouts due to deaths, or interruption of CT or RT. Mean age of the patients was 50.79 ± 11.36 years (range 34-73 years).

There was no significant difference in total scores between pre and post treatment endoscopic findings in gastric and intestinal mucosa ($P > 0.05$). In a subgroup analysis there was only in congestion group a significant difference between pre and post treatment endoscopic findings for gastric mucosa ($P = 0.006$) and intestinal mucosa ($P = 0.02$) but not for other subgroups.

Post treatment endoscopic scores for gastric mucosa and their correlation with RTOG-EORTC scores are shown in Table 3. Overall 13 patients (54.2%) had no endoscopic finding at all but 3 (23%) of them were scored as RTOG-EORTC grade 1 and 5 (38.5%) as grade 2. In addition, 9 patients had an endoscopy score of 1, but 5 were scored as RTOG-EORTC grade 0 and 4 as grade 2. Even in patients with an endoscopy score of 4 the RTOG-EORTC score was 1 or 2. These results showed that there was no correlation between endoscopic and clinical scorings for gastric mucosa.

Post treatment endoscopic scores for intestinal mucosa and their correlation with RTOG-EORTC scores are shown in Table 4. Overall 3 patients (15.8%) with an endoscopic score of 0 were scored as RTOG-EORTC grade 1 and 7 (36.8%) as grade 2 but 9 (47.4%) as 0. One of 4 patients with endoscopic score 1 had a clinical score of 0, another one had a score of 1 and 2 had a score of 2. There was no correlation between endoscopic scoring and RTOG-EORTC scores for intestinal mucosa.

DISCUSSION

The role of adjuvant treatment has been controversial until now in resected gastric cancers^[9-11]. Most of the adjuvant treatments consisted of chemotherapy with a number of phase III trials and three meta-analyses on this subject^[12,13]. Survival advantage with adjuvant chemotherapy is very minor according to these meta-analyses. A randomised trial from the British Stomach Cancer Group^[14] concerning adjuvant radiation alone failed to demonstrate a survival benefit although locoregional failures were decreased from 27% to 10.6%. The recent MacDonald^[1] trial combining adjuvant chemotherapy with radiation showed a major

survival advantage in comparison to only chemotherapy or radiation. In a recent trial by Ajani *et al.*^[15] preoperative chemoradiotherapy increased curative resection rate, overall survival duration and disease-free survival duration. After these publications many centers accepted this chemoradiation treatment protocol as their standard therapy after surgical resection of gastric cancer. But the long-term side effects of these therapies on gastrointestinal mucosa have not been well studied. If we take the 5-year survival rate of 36% and the 3-year survival rate of 50% into consideration^[1], the early detection of side effects becomes even more important.

Gastrointestinal mucositis representing the injury to the gastrointestinal tract is becoming recognised increasingly as a toxicity with many chemotherapy regimens and radiation^[2]. It can be identified all over the alimentary tract. But there is little data in the literature concerning gastrointestinal mucositis especially gastric and intestinal mucositis^[16], whereas esophageal mucositis is the best studied one^[17-19]. There is one study by Sartori *et al.*^[3] describing gastric erosions after chemotherapy with cyclophosphamide, methotrexate and 5-fluorouracil.

In order to assess gastrointestinal mucositis a scoring system is needed which should be objective, validated and reproducible across all clinical conditions. Because of these a number of different clinical scoring systems have been developed, the majority of them measuring oral mucositis like NCI-CTC, WHO, RTOG^[20]. There is no clinical scoring system specially designed for gastric or intestinal damage. The RTOG-EORTC scale measures the late radiation morbidity^[20]. Most of the clinical systems measure acute toxicity and not the permanent damage to the gastrointestinal mucosa. But Yeoh *et al.* have shown that permanent damage occurs in 70%-90% of patients undergoing radiotherapy and because the patients treated for gastric or pelvic cancer constitute the majority of long-term cancer survivors the prevalence of chronic toxicity becomes more important^[21,22]. Moreover some patients may be asymptomatic but still have GI mucositis^[23,24].

Endoscopy is the gold standard of evaluating the gastrointestinal mucosa. Endoscopic scoring systems are very rare in the literature regarding the chemotherapy or radiotherapy induced injury to the alimentary tract. Sartori *et al.*^[3] have conducted a randomized and placebo controlled study evaluating the misoprostol and omeprazole in the prevention of chemotherapy-induced acute gastroduodenal mucosal injury and used an arbitrary endoscopic

score system comprising erosions or ulcers in gastric or duodenal mucosa. They repeated the same study design later with omeprazole and ranitidine and found the score system a useful tool for the detection of gastroduodenal injury after CT^[25]. But both of these studies measured only acute effects (namely 7 d) of CT and did not measure the effects of radiation^[3,25].

Wachter *et al*^[7] conducted a study on late rectal mucosal damage after conformal radiotherapy for prostatic carcinoma and used a six-scaled rectoscopy score for the evaluation of radiation induced proctitis. They concluded that for a valid and reliable correlation and comparison of radiation side effects an accurate objective tool like rectoscopy should be used. Chi *et al*^[26] have developed an endoscopic classification system of chronic radiation induced -proctopathy based on telangiectasia density and vascular coalescence. They found a good reproducibility rate among experienced endoscopists as well as gastroenterology fellows.

Based on these existing endoscopic scoring systems we planned to evaluate the late effects of chemoradiation on gastric and intestinal mucosa. Our study was unique because it evaluated the long-term (1 year) effects of both chemotherapy and radiation and it was not done on intact mucosa as the previous studies but after radical surgery.

The endoscopic score systems proposed by Sartori^[3] was not used because there were no items like stricture or necrosis representing long-term effects in their system. The system proposed by Wachter^[7] took all these parameters into consideration and was based on a terminology prepared by the OMED (World Gastroenterology Association). These terminologies are developed as the Minimal Standard Terminology for data processing in gastrointestinal endoscopy and used widely all over the world by gastroenterologists. Therefore we chose the system of Wachter which is proposed for radiation proctitis and evaluated this endoscopic scoring system on gastric and duodenal mucosa after chemoradiation for locally advanced gastric carcinomas with a resection before. The system was easy to use during routine endoscopy and easy for objective documentation. We could not find any significant difference in total scores between pre and post treatment endoscopic findings in gastric and intestinal mucosa. In a subgroup analysis there was only in congestion group a significant difference between pre and post treatment endoscopic findings for gastric and intestinal mucosa but not for other subgroups. Congestion is the first step in the mucosal damage and is mostly reversible. Therefore we can say that there was no significant chronic mucosal damage after chemoradiation and our endoscopic scoring system is a useful objective method to measure chronic damage. Our study also demonstrated that clinical scoring systems like RTOG-EORTC were not suitable to show gastrointestinal mucositis and there was no correlation between endoscopic and clinical scorings for gastric mucosa because the symptoms used in RTOG-EORTC might be caused by other physiological and individual factors. This observation was confirmed also by Wachter and Koc^[7,19]. Based on this discorrelation between clinical and objective endoscopic scoring systems we propose to evaluate the chronic toxicity of chemoradiation with endoscopy and to decide discontinuation of therapy only after objective findings.

Endoscopic scoring systems are reliable and reproducible tools for the evaluation of gastric and duodenal mucosa after chemoradiation and might be of benefit because they show the gastrointestinal injury much more before it becomes clinically evident and give the best estimation of gastroduodenal mucositis in comparison to widely used clinical scoring systems where symptoms seem to be exaggerated due to multiple confounding variables.

In conclusion, there is no late gastroduodenal damage due to adjuvant chemoradiation in locally advanced gastric adenocarcinomas after operation.

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RAPID COMMUNICATION

Celiac disease in South-West of Iran

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Abstract

AIM: Celiac disease is characterized by life-long gluten intolerance. Clinical features of patients with celiac disease are variable. Studies about the prevalence of celiac disease in our country are scarce and there is no study on the prevalence of celiac disease in southern Iran. In the current study, clinical, laboratory and histological features of 52 patients with celiac disease were evaluated.

METHODS: In a cross sectional study we retrospectively studied the characteristics of 52 celiac patients at Ahwaz JundiShapour University Hospitals (AJSUH) from November 1, 1999 to 1st Sep 2004. Intestinal biopsy and serum antigliadin and anti-endomysium antibodies were used for the diagnosis of patients. Mucosal lesions were classified according to the criteria of Marsh. Antigliadin antibodies were measured with a commercial enzyme-linked immunosorbent assay. Anti-endomysium antibodies were analyzed by indirect immunofluorescence with the use of a section of monkey esophagus. Routine hematological and biochemical analyses and measurement of immunoglobulin levels were undertaken.

RESULTS: Male: female ratio was 1.08. The mean \pm SD patient age was 21 ± 4.5 years (range 10-70 years) and the most common symptoms were diarrhea and weight loss (78.8%) followed by fatigue (73.1%), pallor (65.4%), anorexia (40.4%), abdominal distention (32.7%), and failure to thrive (23.1%). Diarrhea and weight loss and fatigue were the most common findings. Iron deficiency anemia was found in 63.2% of patients and this became normal after adoption of a gluten-free diet in all patients. Immunoglobulin A, IgG antigliadin antibodies and IgA anti-endomysium antibodies were found in 33 and 48 cases, 78.8% and 85.4% of patients, respectively. Biopsy

of the small intestine revealed that 90.4% of patients had typical lesions according to the Marsh classification.

CONCLUSION: Although classical presentation was seen in most of the patients, atypical clinical manifestations of celiac disease should be kept in mind. In particular, patients with uncommon findings, such as short stature, and iron-deficiency anemia, should be screened for celiac disease. Further epidemiological studies in our area in the general population and in high risk groups seem to be indicated.

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Key words: Celiac disease; IgG endomysial autoantibodies; Southern Iran

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INTRODUCTION

Celiac disease (CD) is an autoimmune enteropathy characterized by chronic inflammation of the small intestinal mucosa and the presence of typical auto antibodies. In epidemiological studies the prevalence has been found extremely variable^[1]. The diagnosis can sometimes be difficult due to the wide spectrum of signs and symptoms. Environmental and genetic factors contribute to the clinical presentation of the disease^[2,3]. Prevalence rates of 1:120 to 1:300 have been reported in Western Europe^[4,5]. The clinical picture of the disease includes milder forms and older age at diagnosis^[6]. Screening programs within populations indicate that the disease is underdiagnosed^[7]. Adult celiac patients tend to remain asymptomatic or oligosymptomatic. However, the increased risk of autoimmune diseases and intestinal lymphoma or carcinoma in individuals with CD calls for screening on the slightest suspicion and the disease needs to be treated even when there are no symptoms^[8]. A previous report from Iran indicates an estimated prevalence of up to 1/166 for celiac disease^[9]. To the best of our knowledge few data have been available about the epidemiological features of adult CD in west southern of Iran (Khouzestan).

The aim of this study was to investigate prospectively the clinical picture of diagnosed adult CD in this area.

MATERIALS AND METHODS

Study group

All newly diagnosed cases of CD were registered prospectively from November 1, 1999 to 1st Sep 2004 at Ahwaz JundiShapour University Hospitals (AJSUH). A celiac disease-specific questionnaire was used for data collection and asked for name, birth date and present height and weight, and sought information on intermittent abdominal pain, constipation, diarrhea, known chronic diseases and, associated disorders and family history of celiac disease. Physical examination, blood chemistry, urinalysis, and examination of stool for occult blood, ova and parasites were performed for all patients. If clinically indicated, other para clinical workups such as thyroid function tests, colonoscopy, small bowel study and abdominal ultrasonography were requested. Prior to the diagnostic procedures, informed consent was obtained from all the patients in the study.

CD serology

Total serum IgA level was measured in all subjects to identify IgA deficient cases. IgA antibody titres against gliadin were measured using a commercial enzyme-linked immunosorbent assay (Biosystem, Madrid, Spain). An IgA antibody against gliadin greater than 20 AU/mL was considered positive. Serum IgA antibodies against endomysium were also measured by immunofluorescence (Biosystem).

Diagnostic criteria

Patients who tested positive for IgA antibodies against gliadin or IgA antibodies against endomysium and cases with IgA deficiency underwent upper endoscopy (with Pentax EG2930K endoscopic equipment after an overnight fasting), and six biopsies were taken from the second portion of the duodenum. The biopsy samples were incubated in neutral buffered formalin and processed according to standard procedures. All biopsy specimens were reviewed by two pathologists experienced in celiac disease pathology and graded according to the modified Marsh criteria (10:11).

Patients identified as having CD in biopsy specimens were started on a gluten-free diet. The patients were re-evaluated regularly by repeated IgA antibodies against endomysium serology and repeated upper endoscopy after 6 months. An excellent response was defined as complete elimination of all symptoms. Partial elimination or improvement of some symptoms was considered a good response.

Our study protocol was approved by the ethics committee of the *Ahwaz Jundishapur University of Medical Sciences*.

Statistical analysis

Collected data were coded, analysed and computed, using the Statistical Package for Social Sciences (SPSS) version 10 (SPSS Inc., Chicago, IL, USA). Simple statistics such as frequency, and standard deviation were used. Chi-square and Student's *t*-tests were used for comparison.

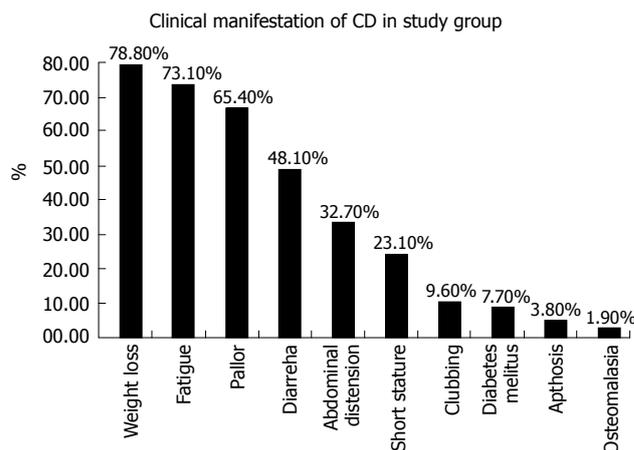


Figure 1 Presenting clinical picture of cases with celiac disease in our study.

RESULTS

A total of 52 patients were identified over the period of our study. Males numbered 27 and females 25 giving a male to female ratio of 1.08. The mean \pm SD patient age was 21 ± 4.5 years (range 10-70 years). Twenty-two of these 52 were adults whereas the remainder were children (< 15 years). Short stature and refractory anemia were the mode of presentation in 12 (54%) and 5 (22.7%) children respectively. The most common symptoms were diarrhea and weight loss (78.8%) followed by fatigue (73.1%), pallor (65.4%), anorexia (40.4%), abdominal distention (32.7%), and failure to thrive (23.1%). Diarrhea and weight loss and fatigue were the most common findings. Anemia was found in 63.2% of patients and this became normal after adoption of a gluten-free diet in all patients (Figure 1).

Anemia was suggestive of iron deficiency in all cases with low serum iron levels and elevated total iron binding capacity. Immunoglobulin A, IgG antigliadin antibodies and IgA anti-endomysium antibodies were found in 33 and 48 cases, 78.8 and 85.4% of patients, respectively. Biopsy of the small intestine revealed that 90.4% of patients had typical lesions according to the Marsh classification. Skeletal radiographs were normal in all patients. Bone mineral densitometry showed osteoporosis in each of the 7 adult patients in which this test was performed (defined as Z score less than -2.0). Follow up data was available in 46 cases. The mean follow up period was 38 mo. During the follow up period there was a significant gain in weight and improvement in symptoms and hemoglobin levels in all the cases. Only 10% (5 cases) had their first-degree relatives screened for the disease and all of them were negative.

DISCUSSION

We performed a prospective study on the newly diagnosed cases of CD in the south-West of Iran 1999-2004. There is a little clinical data available about celiac disease in this area where the disease is considered rare. This is due to lack of a registry system of celiac patients and awareness

of this disease and a delay in physicians reaching the diagnosis of celiac disease. The primary aim of this study was to describe the clinical features at presentation in a group of Iranian patients diagnosed with celiac disease in Khuzestan in west southern of Iran.

The demographic characteristics of adult celiac patients in our area were similar to the clinical features described from populations in New Zealand^[12], South Yorkshire^[13] and Sweden^[14] with a peak in diagnosis in the second decade of life^[15]. Our study shows a female to male ratio of 1.08 a female predominance in keeping with published studies^[11-15]. Twenty-two (42%) of biopsy-proven patients in our study had been diagnosed in childhood (< 15 years). More than 78.8% of patients in this survey presented with diarrhea. This frequency was higher than series of patients from Europe^[16] or Canada^[17]. Diarrhea with or without malabsorption syndrome is regarded as the classical presentation of celiac disease^[18]. In countries where the disease is considered to be common, the percent of patients presenting with diarrhea has been declining. In a study from Edinburgh only 50% presented with diarrhea^[19]. Other presentations have been termed atypical, silent, or sub clinical^[18] and include anemia, bone disease vague GI symptoms, or hypertransaminasemia of unknown origin. The higher rate of patients with the typical or classical presentation in our study suggests decreased awareness of celiac disease and its various atypical presentations among physicians in the south-West of Iran. Many of celiac patients in our study have had a previous diagnosis of irritable bowel syndrome. It is not usual practice to screen these patients for celiac disease. However, this diagnostic group represents a large percentage of patients seen in a gastroenterology practice^[20] and may in fact harbor a large number of undiagnosed celiac patients in our country. In a recent study by Shabazkhani *et al* one hundred and five cases of irritable bowel syndrome patients referred to a university clinic in Tehran, were tested for celiac disease. They found 12 (11.4%) cases with celiac specific antibodies (serum IgA antibodies against gliadin, IgG antibodies against gliadin and antibodies against endomysium), of which 11 (10%) proved to have celiac disease confirmed by duodenal biopsy. The authors suggested that all patients suffering from irritable bowel syndrome should be screened for celiac disease in Iran^[21]. We have noted a long duration of symptoms before diagnosis (mean of 3.5 year in our study) this was mostly due to a delay in reaching the diagnosis of CD and a previous diagnosis of irritable bowel syndrome in our cases.

A plausible explanation would be that physicians regard adult celiac disease as rare and fail to consider it in clinical situations other than the classical chronic diarrhea and malabsorption.

A repeated duodenal biopsy to demonstrate improvement of villous atrophy on gluten free diet is advisable for confirmation of diagnosis in CD^[22]. All the cases with CD in our study had a good response to GFD, thus obviating the need for a repeat biopsy to demonstrate improvement of duodenal villous atrophy. An association between celiac disease and autoimmune disorders, such as type I diabetes, autoimmune thyroid disease, and Sjögren's syndrome, has been well documented in the literature^[23].

Table 1 Frequency (%) of associated disorders in study group

Disorders	(n = 52)	%
Inflammatory bowel disease	8	15.3
Thyroid disease	6	11.6
Diabetes mellitus type 1	4	7.7
Collagen vascular disease	3	5.7
Total	21	40.3

We found a higher frequency of associated diseases (40.3%) than in most other studies (10%-15%)^[23], that could be due to the increasing age at diagnosis (Table 1). In our study, the percentage of celiac patients who had a diagnosis of inflammatory bowel disease and thyroid disease were 15.3% and 11.6%, respectively, compared to estimates of 3%-5% and 3% reported in the literature^[24]. Dermatitis herpetiformis and IgA deficiency are relatively common extraintestinal manifestations of celiac disease, but no patients were noted to have such extraintestinal manifestations in our cases. Our survey confirms the increased incidence of small bowel malignancies, adenocarcinoma, and EATCL, in celiac disease^[25]. The malignancies of two cases (male) were lymphoma and occurred in the jejunum. The patients presented with bowel obstruction, and anemia. These two cases had a long history of symptoms of celiac since childhood. Adherence to a gluten-free diet reduces the risk of developing malignancies^[26]. Therefore, earlier diagnosis may have prevented the development of malignancy. We don't know how well the patients with lymphoma had adhered to the diet from the time of celiac diagnosis to the time of lymphoma diagnosis. About 8% of patients in our study were diagnosed with celiac disease at age 40. Men were more prominent in this group. These patients also had a long duration of symptoms. There was a trend toward more hip fractures in patients diagnosed at age 40 compared to those diagnosed at age < 40. Earlier diagnosis may prevent the development of osteoporosis and subsequent fractures^[27]. There was a high frequency of metabolic bone disease and secondary hyperparathyroidism (up to 50%) in a cohort that was reported in untreated celiac disease cases from Turkey^[28]. Prevalence of metabolic bone disease in Iranian patients with CD is not known. Bone mineral densitometry showed osteoporosis in each of the 7 adult patients in which this test was performed (defined as Z score less than -2.0). These data underscore the need for adding calcium and vitamin D supplementation to gluten free diet even though they may be asymptomatic or show no biochemical or radiological evidence of bone disease. In summary, our study provides data about the clinical features of celiac disease in our area in south-West of Iran. The majority of patients who presented with diarrhea had a long duration of symptoms before diagnosis and we considered the diagnosis delayed. Celiac disease may not always present with classical clinical features so the possibility of this disease should be kept in mind. Celiac disease may result in a markedly increased risk for the development of small bowel malignancies and earlier diagnosis is very important. With the widespread

availability of screening tests for celiac disease, identifying cases in at risk groups by screening should also be considered.

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RAPID COMMUNICATION

Immunogenicity of recombinant hepatitis B vaccine in treatment-naïve and treatment-experienced chronic hepatitis C patients: The effect of pegylated interferon plus ribavirin treatment

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Abstract

AIM: To retrospectively evaluate the vaccination-induced anti-HBs seroconversion rates in treatment-naïve and treatment-experienced chronic hepatitis C (CHC) patients. Also to prospectively evaluate the seroconversion rates in CHC patients during pegylated interferon (PEG) plus ribavirin (RIB) treatment.

METHODS: Seventy treatment-naïve CHC patients (group A), 22 sustained virological responders-SVR following interferon (IFN) plus RIB treatment CHC patients (group B) and 121 healthy subjects (group C) had been participated in the same HBV vaccination schedule (20 µg, 0-1-6 mo). Seroconversion was considered if anti-HBs levels were above 10 mIU/mL within 3 mo following the third dose of the vaccine. Moreover, we prospectively selected 30 non-cirrhotic CHC patients and evaluated them for the efficacy of the same vaccine schedule randomizing them in two groups: Group-1, 15 CHC patients received the first dose of the vaccine in parallel with the initiation of PEG plus RIB treatment and Group-2, 15 patients received the same vaccination schedule without concomitant treatment. Determination of anti-HBs was performed at mo 1, 2, and 7. Statistical analysis of data was based on ANOVA student's *t*-test and chi-square analysis ($P < 0.05$).

RESULTS: Fifty-eight of 70 group A patients (82.85%), 20/22 group B (90.9%) and 112/121 healthy subjects (92.56%) had been seroconverted. The seroconversion rates were significantly higher in the control group than in treatment-naïve CHC patients ($P = 0.04$). The

corresponding rates were comparable between group A and group B CHC patients ($P = 0.38$). The vast majority of non-responders (10/14, 71.43%) had been infected by genotype-1 of HCV. The seroconversion rates were comparable between group 1 and 2 CHC patients at mo 1 (20% versus 26.7%, $P = 0.67$), mo 2 (46.7% vs 60%, $P = 0.46$) and mo 7 (86.7% versus 93.3%, $P = 0.54$) of follow-up.

CONCLUSION: The immunogenicity of HBV vaccine seems to be lower in CHC patients compared to healthy subjects. SVR following IFN plus RIB treatment does not affect the antibody response to HBV vaccine. Infection by genotype-1 seems to negatively influence the seroconversion rates. Vaccination against HBV during PEG plus RIB combination treatment is not beneficial in terms of anti-HBs seroconversion rates.

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Key words: Hepatitis B vaccine; Chronic hepatitis C; Interferon; Ribavirin

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INTRODUCTION

Hepatitis B virus (HBV) and hepatitis C virus (HCV) are two of the most common causes of chronic liver disease and hepatocellular carcinoma in the world. Both viral infections share many of the same routes of transmission, including parenteral exposure, promiscuous sex and vertical transmission^[1,2,3]. HBV infection can be prevented by the administration of a safe and immunogenic vaccine^[4,5,6] whereas no vaccine exists until now for HCV infection due to the high variability of the virus and its subsequent ability to escape immune surveillance.

Dual infection by HBV and HCV is clearly associated with more severe liver disease than infection by a single virus, whereas acute HBV infection in patients with chronic HCV-related liver disease is associated with a severe and often fulminant course of the disease^[7,8,9]. Hepatitis B vaccination is recommended for patients with chronic HCV infection without immunity to HBV^[10] in a cost-effectiveness approach^[11]. Several studies suggest that the immunogenicity of recombinant hepatitis B vaccine is decreased in patients with chronic hepatitis C, compared to healthy controls^[12,13], especially in those with advanced liver disease^[14]. In only one study from Taiwan, was the antibody response to hepatitis B surface antigen (anti-HBs) similar in chronic hepatitis C patients and healthy subjects, who were both susceptible to HBV infection, after the first, second and third dose of the vaccine^[15]. HCV viral load does not seem to correlate with the vaccination-induced anti-HBs response in chronic HCV infected patients vaccinated against HBV^[12,13] whereas the main predictive factors for non-response to HBV vaccine seem to be age (> 50 years), body weight (> 75 kg) and the presence of cirrhosis^[12,14]. The ideal time for HBV vaccination and the optimum HBV vaccination schedule (dose/interval) of chronic HCV infected patients have not been defined yet.

To our knowledge data about the impact of antiviral-immunomodulatory treatment against HCV infection on the safety and efficacy of the recombinant HBV vaccine are limited^[16]. Moreover, there are no data about the impact of pegylated-interferon alpha (PEG-IFN α) plus ribavirin (RIB) treatment in the vaccination-induced anti-HBs response rates in chronic HCV infected patients. In our study we retrospectively evaluated the vaccination-induced anti-HBs response rates in treatment-naïve chronic HCV infected patients and in chronic HCV infected patients who exhibit sustained virological response after interferon-alpha plus ribavirin treatment, in comparison with healthy-HBV susceptible subjects vaccinated against hepatitis B with available data. The second aim of the study was to prospectively evaluate the vaccination-induced anti-HBs response rates in a group of chronic hepatitis C patients during PEG-IFN α plus RIB treatment in order to gain information about the optimum period of HBV vaccination.

MATERIALS AND METHODS

The study was performed at the Hepatology Unit of Hippokraton Hospital of Athens, Greece. In this study we retrospectively analyzed 92 patients with serologically, virologically and histologically confirmed chronic hepatitis C (CHC) and 121 healthy subjects, with available data from medical records between October 2000 and October 2004, for the immunogenicity of recombinant hepatitis B vaccine, at a regular dose (20 μ g) and classical vaccination schedule (0, 1, 6 mo). Seventy CHC patients had never experienced treatment for hepatitis C before (group A) and 22 CHC patients of the study population had been treated with interferon-alpha plus ribavirin and they had been characterized as sustained virological responders (group B), according to virological data. Sustained virological

response (SVR) was confirmed by undetectable serum HCV-RNA at the end of treatment and again six months after completion of treatment, as well as just before the beginning of the vaccination schedule. Healthy subjects with available data who had participated in the vaccination schedule were characterized as control group (group C).

All participants of the study population had their height and weight recorded at the beginning of the study and their body mass index (BMI) was calculated by dividing patient's weight (kg) by the squared height (m²). Patients had also been queried about their drinking behavior, so an individual who had been drinking at least 30 gm of alcohol per day for at least 5 years was considered as an alcoholic and was excluded from the study. Patients excluded from our study were also those with histologically confirmed or clinically decompensated liver cirrhosis, transplanted HCV infected patients or those on waiting list for liver transplantation, patients with chronic renal failure, HIV-positive patients, patients with solid tumors or hematological malignancies, immunosuppressed patients in general or those who had ever received immunosuppressive treatment. Pregnant women and patients under 18 or over 70 years of age, as well as people, who had already been exposed to hepatitis B virus as confirmed by their serological status, were also excluded.

Routine biochemical and hematological tests were performed using automated techniques. Hepatitis B surface antigen (HBsAg), antibody to hepatitis B surface antigen (anti-HBs), and total antibody to hepatitis B core antigen (anti-HBc) as well as antibody to human immunodeficiency virus (anti-HIV) and antibody to hepatitis C virus (anti-HCV) were detected using routine commercially available enzyme immunoassays (Abbott Laboratories, Abbott Park, Illinois, USA). Serum HCV-RNA levels were measured in anti-HCV positive patients by a commercially-available quantitative polymerase chain reaction (PCR) assay (Cobas Amplicor HCV test, version 2, Roche Diagnostics, Branchburg, New Jersey, USA) and HCV genotype was defined in HCV-RNA positive patients using INNOLIPA HCV assay (Innogenetics, Belgium).

Liver biopsy was performed in CHC patients according to Menghini technique. Eligible anti-HCV positive/HCV-RNA positive patients had at least two available biochemical determinations of serum alanine transaminase (ALT) levels being above 60 IU/L (> 1.5 times the upper limit of normal) and had no contraindication to liver biopsy. Patients were excluded from liver biopsy if they had a serious medical condition (heart failure, renal failure, uncontrolled hypertension or diabetes mellitus *etc*), or elevated bleeding risk (platelets < 100 000/mm³, prothrombin time \geq 16 s, treatment with heparin or coumadin, aspirin or non-steroidal anti-inflammatory drugs in the past 8 d). Liver tissue was fixed in 10% formalin and paraffin-embedded sections were stained with hematoxylin-eosin and trichrome stains. A single experienced hepatopathologist, who was blinded to clinical markers other than HCV-antibody status, evaluated all biopsy specimens that were scored according to Ishak scoring system (grade = 0-18, stage = 0-6)^[17]. As previously mentioned, CHC patients with histologically confirmed

cirrhosis were excluded from the study.

All patients and healthy subjects (controls) received 20 µg of recombinant DNA vaccine for hepatitis B (Engerix-B, GSK) intramuscularly (deltoid region) at three different time intervals (0, 1, 6 mo). Determination of anti-HBs antibodies was performed in all individuals of the study population within 3 mo following the third dose of the vaccine. Seroconversion was considered if anti-HBs levels were above 10 mIU/mL and non-reagent response if anti-HBs levels were below 10 mIU/mL.

Moreover, we prospectively selected 30 non-cirrhotic CHC patients and evaluated them for the efficacy of the same vaccine, using the same dose (20 µg) and treatment schedule (0, 1, 6 mo) randomized them (1/1 randomization) in two groups. In group-1, 15 patients received the first dose of the vaccine in parallel with the initiation of pegylated interferon-α2a plus ribavirin treatment, whereas in group-2, 15 patients received the same vaccination schedule without concomitant treatment for chronic hepatitis C. All group-1 patients were treated with fixed dose of pegylated interferon-α2a (180 µg/wk) and genotype-related ribavirin dose (800 mg/d for genotype 2/3 and 1000-1200 mg/d for genotype 1/4-infected patients, depending on baseline body weight < or ≥ 85 kg, respectively). The duration of treatment was genotype-based (24 wk for genotype 2/3 infected patients and 48 wk for genotype 1/4-infected ones). Determination of anti-HBs antibodies was performed in all CHC patients from both groups at baseline as well as one month following every dose of the vaccine (at mo 1, 2, and 7) and seroconversion was also considered if anti-HBs levels were above 10 mIU/mL.

The study conformed to the ethical guidelines of the 1975 Declaration of Helsinki. Written informed consent forms were obtained from the participants in the prospective schedule of the study before the liver biopsy procedure.

Statistical analysis of data was based on ANOVA student's *t*-test and chi-square analysis (χ^2). The results are presented as mean ± SD. A probability value of less than 5% ($P < 0.05$) was considered significant.

RESULTS

Group A, group B and group C participants of the study population were age, sex and BMI matched [age: 27.1 ± 4.0 versus 27.8 ± 4.4 versus 26.7 ± 4.5, respectively, $P = 0.63$, sex (male/female): 44/26 versus 14/8 versus 63/58, respectively, $P = 0.52$, BMI: 22.46 ± 1.6 versus 22.05 ± 0.8 versus 23.01 ± 1.4, respectively, $P = 0.43$]. Twenty seven of the 70 chronic HCV-infected patients from group A (38.57%) and 10 of 22 from group B (45.45%) were prior injecting drug users (IDU) whereas 13 patients from group A (18.57%) and 4 from group B (18.18%) had post-transfusion hepatitis (PTH) C. The rest of chronic hepatitis C patients from both groups had the cryptogenic form of transmission of HCV infection. Twenty five of 70 group-A patients (35.71%) and 9 of 22 group B ones (40.9%) had been infected by genotype-1 of HCV. The remaining 58 chronic HCV-infected patients of the study population had been infected by genotype-3 (50/58,

Table 1 Epidemiological, virological and histological baseline data of group-1 and group-2 CHC patients of the study population

	Group 1 (n = 15)	Group 2 (n = 15)	P-value
Age (yr)	26.9 ± 3.8	27.3 ± 4.1	0.510
Sex (male/female)	8/7	9/6	0.713
BMI (kg/m ²)	22.45 ± 1.8	22.12 ± 0.9	0.390
HCV-RNA (-log ₁₀ IU/mL)	5.32 ± 0.67	5.24 ± 0.52	0.765
Grade (0-18)	5.6 ± 2.3	4.2 ± 1.5	0.089
Stage (0-6)	1.3 ± 0.5	1.5 ± 0.7	0.276
Genotype (1/non-1)	3/12	2/13	0.624

86.2%) or 2 (8/58, 13.8%) of HCV.

Fifty-eight of the 70 group A patients (82.85%), 20 of the 22 group B ones (90.9%) and 112 of 121 healthy subjects (92.56%) had been seroconverted (anti-HBs ≥ 10 mIU/mL) within three months following the third dose of the vaccine. The vaccination-induced seroconversion rates were significantly higher in the control group than in treatment-naïve CHC patients ($P = 0.04$) whereas the corresponding rates were comparable between group A and group B CHC patients ($P = 0.38$). Overall, among the 92 CHC patients who had been evaluated, 78 (84.78%) had been seroconverted to anti-HBs, a percentage significantly lower than the corresponding one from the control group ($P < 0.05$). The finding that among the 14 non-responders to vaccination schedule chronic HCV-infected patients from both groups (12 treatment-naïve and 2 SVR's), the vast majority (10/14, 71.43%) had been infected by genotype-1 of HCV seems very important and needs further investigation.

Table 1 shows the epidemiological, virological and histological baseline data of group-1 and group-2 CHC patients of the study population. The two groups were comparable for all baseline parameters (age, sex, BMI, viral load, HCV-genotype, grade and stage of liver disease) before the initiation of the vaccination schedule, with (group-1) or without (group-2) concomitant antiviral-immunomodulatory treatment. The anti-HBs antibody response rates were comparable between these two groups of CHC patients at mo 1 (20% vs 26.7%, $P = 0.67$), mo 2 (46.7% vs 60%, $P = 0.46$) and mo 7 (86.7% vs 93.3%, $P = 0.54$) of follow-up, as shown in Figure 1.

DISCUSSION

The main findings of our study were that chronic HCV infected patients exhibit a lower response to recombinant HBV vaccine compared to healthy subjects, using the recommended dose (20 µg) and the typical vaccination schedule (0, 1, 6 mo), irrespective of the virological response to prior interferon-alpha plus ribavirin treatment. Moreover, we found that concomitant pegylated interferon-alpha plus ribavirin treatment does not influence the antibody response to HBV vaccine in chronic HCV infected treated patients.

Factors known to affect HBV vaccine immunogenicity include age, sex, BMI and smoking^[18]. In our study all

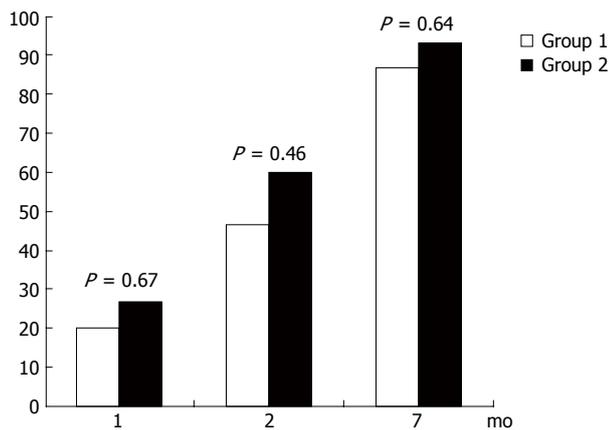


Figure 1 Anti-HBs antibody response rates between group-1 and group-2 CHC patients at one month following every dose of the vaccine (mo 1, 2, and 7).

these parameters except for the smoking behavior, which had not been taken under consideration, were comparable between the patients and the control group of the study population. Occult HBV infection (confirmed as serum HBV-DNA positivity by a sensitive PCR assay in the absence of serological markers of HBV infection) is considered to be associated with the absence of response to recombinant HBV vaccine^[19]. Cacciola *et al* reported that chronic HCV infected patients exhibit a particularly high incidence of occult HBV infection^[20], which means that the absence of serum HBV-DNA testing in our study population possibly represents a limitation of our study. On the other hand, the results of our study reflect the expected immunogenicity of HBV vaccine in chronic HCV-infected patients in the current clinical practice where HBV seronegative, chronic HCV-infected patients, are not routinely tested for serum HBV-DNA.

Hyporesponsiveness to HBV vaccine in chronic HCV infected patients had been previously demonstrated by other studies, especially in cirrhotic patients^[12,13,14]. Several factors can explain the reduced cellular and humoral arms of immune response in these patients, including the lymphocytopenia and the altered composition of the T-lymphocyte subpopulation in the peripheral blood, the inappropriate interaction between antigens, T-cells and T-cell receptor/major histocompatibility complex molecule, and the impaired proliferative response to T-cell activation by various mitogens^[14]. Liver cirrhosis, confirmed either by liver biopsy or signs of decompensation (jaundice, ascites, variceal bleeding, *etc*) was one of the exclusion criteria of our study population, suggesting that chronic HCV infected patients exhibit a lower response to HBV vaccine even in the absence of severe liver disease. A possible impairment in both humoral and cellular-mediated immune responses in non-cirrhotic chronic HCV-infected patients has been suggested to explain this low response to HBV vaccination in such individuals^[13, 21].

HCV viral load does not seem to correlate with the vaccination-induced anti-HBs response in chronic HCV infected patients vaccinated against HBV^[13], a finding that was also observed in our study. Sustained virological response following interferon-alpha plus ribavirin combination treatment was not related to statistically

significant increase in anti-HBs seroconversion rates compared to untreated chronic HCV-infected patients in our study. On the other hand, there are some studies that suggest a negative correlation between viral load of HCV and antibody response to HBV vaccination^[12], considering that detection of leukocytes, monocytes/macrophages and B-lymphocytes HCV genomic sequences in chronic HCV-infected patients^[22] could possibly influence the immune response to HBV vaccine. The results of our study suggest that the sustained absence of viremia following interferon plus ribavirin combination treatment is possibly not enough to positively influence the impaired immune response of chronic HCV-infected patients. In concern to HCV genotype, there is only one study that showed a worse response to HBV vaccine in patients who had been infected by genotype-1 of HCV as compared to those infected by genotype 2 or 3^[23], suggesting that in genotype-1 infected patients a different HBV vaccination schedule with possibly higher doses and shorter intervals between the vaccine doses should be used in order to obtain a better response. As previously noted the majority of non-responders to vaccine of the chronic HCV-infected patients of our study population (71.43%) had been infected by genotype-1 of HCV, a finding that needs further investigation.

There is currently no consensus on the optimal HBV vaccination schedule for chronic HCV-infected patients, especially in those who need antiviral-immunomodulatory treatment. Nowadays pegylated interferon-alpha plus ribavirin represent the initial treatment of chronic hepatitis C patients. Interferon-alpha has been previously used as a co-stimulant in non-responders to primary HBV vaccination schedules^[24]. Chlabicz *et al* published a study that did not demonstrate any beneficial or detrimental effect of interferon-alpha on HBV vaccine immunogenicity^[16]. Moreover, this study suggests that vaccination against HBV during treatment with interferon-alpha is safe and well tolerated. It should be noted however that, in this study, the interferon-treated group was small and the patients had received varying numbers of interferon doses as well as different interferon types ($\alpha 2a$, $\alpha 2b$). In our study we evaluate the HBV vaccination induced anti-HBs seroconversion rates in chronic HCV-infected patients during fixed dose pegylated interferon-alpha plus ribavirin combination treatment. We found no differences in the anti-HBs seroconversion rates between the treatment-naïve and the treatment-experienced group of chronic HCV-infected patients; this finding needs further confirmation in prospectively scheduled large-scale controlled studies.

In conclusion, the immunogenicity of the recombinant HBV vaccine seems to be lower in chronic HCV-infected patients as compared to healthy subjects. Sustained virological response following interferon plus ribavirin combination treatment does not affect the antibody response to HBV vaccine in chronic HCV-infected patients, whereas infection by genotype-1 of HCV seems to negatively influence the anti-HBs seroconversion rates. Vaccination against HBV during pegylated interferon-alpha plus ribavirin combination treatment is not beneficial in terms of anti-HBs seroconversion rates, as compared

to treatment-naïve HBV-vaccinated chronic HCV-infected patients.

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Primary gastric melanoma: A case report

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Abstract

Melanoma accounts for 1-3 per cent of all malignant tumors. Except cutaneous, other less common melanomas include, among others, those in the GI tract. However, their primary or secondary nature is often difficult to establish. Referring to the stomach, scattered cases of primary melanomas have been reported in the literature.

We report a case of a man with an ulcerated submucosal mass at the antrum of the stomach, manifested with dull upper abdominal pain, nausea, vomiting, fatigue and anemia. This lesion was histologically proved to be melanoma. A detailed clinical and laboratory investigation revealed no primary site elsewhere.

To our knowledge, very few cases of primary gastric melanoma have been reported. Our case is the fourth ever published and the first located at the antrum of the stomach. The debate upon the primitive nature of such lesions still persists. Thus, specific diagnostic criteria have been proposed.

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Key words: Primary gastric melanoma; Manifestations; Diagnosis

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INTRODUCTION

Primary gastrointestinal malignant melanoma is a very

rare entity. In contrast, metastatic melanoma to the gastrointestinal tract is found during diagnostic workup in 1%-4% of patients with a cutaneous primary and up to 60% of melanoma patients in autopsy^[1-4]. Several reports have addressed the debate on whether primary gastrointestinal melanoma actually exists by proposing certain diagnostic criteria^[5,6].

Primary melanomas located in the stomach are extremely rare. Although the cell of origin has not been identified since normal stomach epithelium lacks melanocytes, possible etiologies for the occurrence of primary melanoma have been described. Ectopic migration of melanocyte precursors or differentiation of the APUD cells to melanocytes has been advocated as possible mechanism for the development of malignant melanoma^[7,8]. Furthermore, benign melanosis of the stomach has been reported in association with melanoma of the esophagus documenting the presence of melanocytes in the stomach, rarely.

We report on a case of an ulcerated submucosal mass at the antrum of the stomach, which was histologically proved to be a melanoma. A detailed clinical and laboratory investigation revealed no primary site elsewhere.

CASE REPORT

A 58-year old male was referred to our clinic with a short history of nonspecific symptoms, including nausea, vomiting, dull upper abdominal pain and fatigue, aggravating in intensity over the last 24 h. He had a weight loss of 8 kg over the preceding 3 mo. Clinical examination was unremarkable and laboratory examination showed anemia (Ht: 28%). A fecal occult blood test was positive. Colonoscopy was negative. Endoscopic examination of the upper GI tract revealed a submucosal mass with ulceration at the antrum, 3 cm × 5 cm in size. Biopsy, conducted during endoscopy, revealed a malignant melanotic lesion. Gastric mucosa proved to be infiltrated, by numerous pleomorphic tumor cells with melanin deposits (Figure 1).

At exploratory laparotomy a melanotic lesion was found at the antrum with numerous melanotic lesions identified at the greater omentum (Figure 2). Subsequently, a subtotal gastrectomy with splenectomy was carried out.

Pathological report on the surgical specimen proved the melanotic nature of the tumor. The gastric malignant melanoma was extending through the muscularis propria without invading the serosa. The excised lymph nodes were examined and found negative for metastases and complete excision of the ulcerated lesion was reported. Deposits of

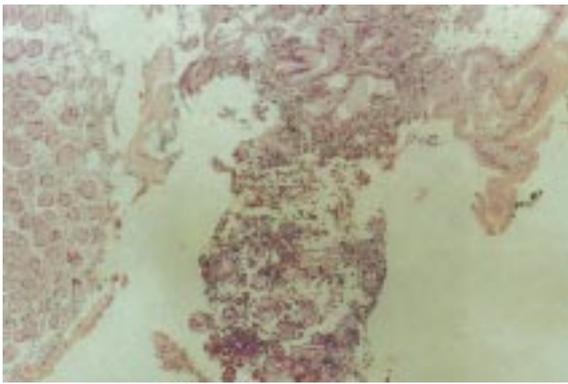


Figure 1 Primary malignant melanoma of the stomach. Gastric mucosa also viewed (HE, $\times 40$).

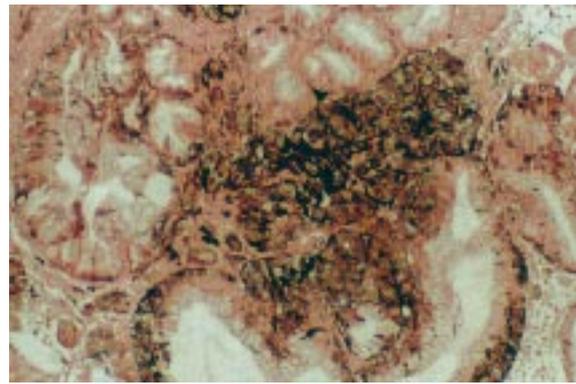


Figure 3 Primary gastric melanoma stained with Masson-Fontana. Plenty of granules of melanin are viewed ($\times 100$).

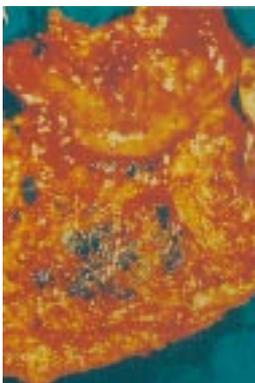


Figure 2 Surgical specimen of a subtotal gastrectomy and splenectomy for a primary melanoma located at the antrum of the stomach. Certain deposits of malignant melanocytes are viewed in the co-excised greater omentum.



Figure 4 Primary gastric melanoma stained with HMB-45 ($\times 100$).

malignant melanocytes, accumulated after migrating from the antrum to the greater omentum through the extensive vascular and lymphatic vessels, were ascertained in the co-excised omentum. Microscopic evaluation showed nests and sheets of epithelioid cells stained positive with Fontana-Masson (Figure 3). Immunohistochemical examination revealed a positive reaction with S-100 protein and HMB-45 antibodies (Figure 4).

The postoperative clinical investigation, as well as the follow-up did not reveal any other primary site. Ophthalmologic and dermatologic examinations were negative. In addition, anoscopy, chest CT and small-bowel barium contrast radiography were also negative for a primary site of melanoma.

The patient denied any further adjuvant therapy. Sixteen months after the operation he is disease-free, as follow-up endoscopic examination of the upper GI tract and abdominal CT revealed no signs of recurrence.

DISCUSSION

Non-cutaneous melanoma represents a rare form of melanoma. In a review of 84 836 cases of melanoma between 1985 and 1994, 91.2% were cutaneous, 5.2% ocular, 1.3% mucosal and 2.2% of unknown primary^[9]. Referring to GI mucosal sites, melanoma has been reported to arise in esophagus, anorectum and small intestine^[10-12]. Moreover, scattered cases of primary melanoma arising in the stomach have also been reported^[5,6,13].

The proposed primary sources, from which GI

melanomas are derived, are melanoblastic cells of the neural crest, which migrate to the ileum through the omphalomesenteric canal or APUD cells, which undergo neoplastic transformation^[14]. Although, there have been no reports on benign melanocytes in normal gastric epithelium, melanosis of the gastric mucosa in association with esophageal and anal malignant melanoma has been reported^[15]. Furthermore, the rare presence of melanosis of the esophageal mucosa is accounted for the occurrence of primary esophageal melanoma^[16]. Thus, it seems reasonable that primary gastric melanoma should be considered possible.

In order to avoid running the risk of misinterpretation of a metastasis to the gastric wall to primary lesion, several reports have issued criteria for the diagnosis of primary melanoma. Suggestive criteria of the primary nature of gastric melanoma include lack of concurrent or previous removal of a melanoma or atypical melanotic lesion from the skin, lack of other organ involvement and in-situ change in the overlying or adjacent GI epithelium. The latter, recognized histologically by the presence of atypical melanotic cells in the basal layer of the epithelium and extending in a "pagetoid" fashion into the more superficial epithelium, may be reported in 40%-100% of primary GI melanomas^[14,17]. Additionally, disease free survival of at least 12 mo after curative surgical excision of the involved organ has been proposed as a criterion for the distinction of a primary lesion from metastatic since 50% of patients

with stage IV melanoma or visceral disease from unknown primary will have died at 12 mo from diagnosis^[9,10].

Etiology of primary GI melanomas remains undefined. No predisposing factor has ever been proposed. In contrast, the rarity of these lesions is easily justified by the paucity of melanocytes in the gastrointestinal tract and the inherent protection from etiologic factors such as ultraviolet radiation^[10].

Manifestations of primary gastric melanoma include anemia and weight loss. Barium contrast radiography and endoscopy have some role in the initial diagnostic procedure. CT scan of the abdomen may reveal a gastric mass with or without evidence of lymph node metastases. Preoperative diagnosis is seldom made because of the non-specificity of the symptoms, including nausea, vomiting, abdominal pain, weight loss and anemia^[18]. Tissue sampling during endoscopy may provide the definitive diagnosis. The presence of pigmentation of an ulcer is the most common endoscopic finding. Immunohistochemical staining with HMB-45 and S-100 will confirm the presence of malignant melanocytes in the mucosa^[19].

Melanomas that arise on mucosal surfaces appear to be more aggressive and are associated with worse prognosis than cutaneous melanomas. The poorer prognosis may be related to delay in diagnosis, an inherently more aggressive behavior of mucosal melanomas or earlier dissemination because of the rich lymphatic and vascular supply of the GI tract mucosa^[10]. However, early detection of primary gastrointestinal melanomas and treatment with curative surgical excision can provide long-term disease free interval^[13].

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CASE REPORT

Xanthogranulomatous cholangitis causing obstructive jaundice: A case report

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or magnetic resonance imaging show an invasive tumor around the bile duct, the tumor should be diagnosed as malignant. The correct diagnosis is difficult when there is a discrepancy between the imaging and cytologic diagnosis. The sensitivity of bile cytology to detect malignancy is too low to make a conclusive diagnosis. We present a case of bile duct stricture due to xanthogranulomatous cholangitis where a cytological diagnosis of class V adenocarcinoma was made preoperatively. Microscopically, the bile duct was surrounded and narrowed by a xanthogranulomatous lesion, but no xanthogranulomatous cholecystitis (XGC) was seen.

Abstract

This article reports the case of a 34-year-old woman with xanthogranulomatous cholangitis who developed obstructive jaundice. Microscopically, the bile duct was surrounded and narrowed by a xanthogranulomatous lesion, but no xanthogranulomatous cholecystitis was seen. Although percutaneous cholangiograms done via the transhepatic biliary drainage showed smooth narrowing of the upper to middle bile duct, the cytology of bile was diagnosed as class V adenocarcinoma. Therefore, right extended hepatectomy and extrahepatic bile duct resection were performed. The differentiation of benign and malignant strictures at the hepatic hilum is often difficult. Xanthogranulomatous cholangitis is one possible diagnosis of a bile duct stricture. Precise review of all the preoperative information is required to make a correct diagnosis.

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Key words: Xanthogranulomatous cholangitis; Obstructive jaundice; Bile cytology; Bile duct stricture

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<http://www.wjgnet.com/1007-9327/12/4428.asp>

INTRODUCTION

The differential diagnosis of bile duct strictures at the hepatic hilum is often difficult. When computed tomograms

CASE REPORT

A 34-year-old woman visited a neighborhood doctor because of jaundice in January 2003. The serum total bilirubin concentration was 7.5 mg/dL. The serum alkaline phosphatase and γ -glutamyltranspeptidase concentrations were 1588 IU/L and 286 IU/L, respectively. There was no leukocytosis. The serum carcinoembryonic antigen, carbohydrate antigen 19-9, and α -fetoprotein levels were within normal limits. Percutaneous transhepatic cholangiodrainage (PTCD) was performed. The cholangiogram showed smooth stenosis of the common bile duct and both hepatic ducts (Figure 1). Bile cytology specimens from the PTCD revealed some cohesive groups of columnar cells with partial papillary growth (Figure 2A). The nucleocytoplasmic ratio was increased and variously sized round nuclei with granular chromatin and prominent nucleoli were observed (Figure 2B). The cytology of the bile was diagnosed as class V adenocarcinoma. She was transferred to our hospital for surgical treatment in February 2003. Computed tomograms of the abdomen demonstrated ascites, but no tumor mass was detected around the bile duct. A diagnostic laparoscopic examination revealed no peritoneal deposits. After percutaneous transhepatic embolization of the right portal vein, extended right hepatectomy and extrahepatic bile duct resection were performed. On inspection, the resected specimen showed wall thickening of the biliary tree from the hepatic duct to the common bile duct. However, the surface mucosa of the extrahepatic bile duct and gallbladder was smooth with no evident neoplastic change. Neither gallstones nor biliary sludge were seen. The cut surface of the bile duct showed a yellowish xanthomatous lesion expanding from the lamina propria toward the serosa (Figure 3). Microscopically, there was fibrous thickening and marked infiltration of foamy



Figure 1 Percutaneous transhepatic cholangiograms showing a stricture of the common bile duct and bilateral hepatic ducts, although the bile duct wall was smooth.

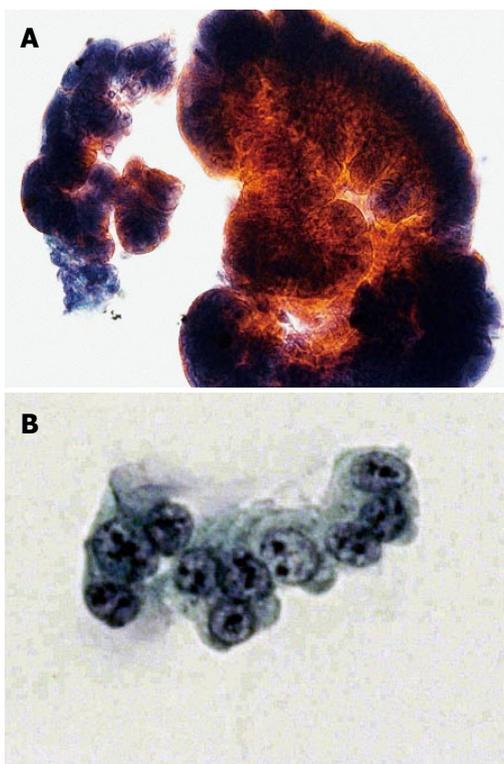


Figure 2 Microscopic cytology showing atypical columnar cells with variously-sized round nuclei with granular chromatin and prominent nucleoli. A: Papanicolaou stain, $\times 200$; B: Papanicolaou stain, $\times 400$.

macrophages, lymphocytes, plasma cells, and eosinophils in the lamina propria of the bile duct mucosa (Figure 4). These fibrotic, inflammatory, and xanthogranulomatous changes were localized to the extrahepatic bile duct sparing the gallbladder, and resulted in luminal narrowing of the bile duct. The gallbladder showed mild infiltration of lymphocytes and plasma cells, thickening of the smooth muscle layer, and edema of the subserosa. Although the surface epithelia of the bile duct showed some hyperplastic



Figure 3 Gross features of the resected specimen showing the absence of a neoplastic lesion, with smooth mucosa in the right hepatic lobe and extrahepatic bile duct.

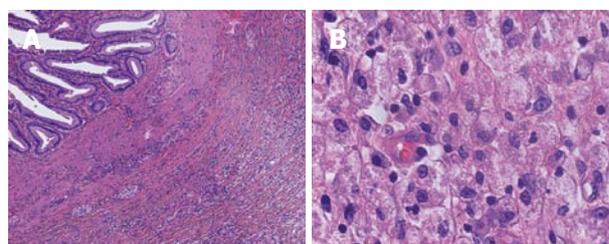


Figure 4 Microscopic view showing marked infiltration of foamy macrophages, lymphocytes, plasma cells, and eosinophils in the lamina propria of the bile duct mucosa. A: HE $\times 200$; B: HE $\times 400$.

features, namely increased luminal folds, no significant structural or cellular atypia were noted. A diagnosis of xanthogranulomatous cholangitis without cholecystitis was made.

DISCUSSION

XGC is a relatively rare inflammatory disease of the gallbladder. Xanthogranulomatous cholangitis is rare, though there are some reports of bile duct stenosis due to extension of XGC^[1,2]. The pathogenesis of XGC remains elusive; cystic duct obstruction with gallstones and bile stasis are thought to be important etiologic factors^[3,4]. In our case, there was no stone in the gallbladder or the bile duct, and XGC was never seen microscopically, although mild inflammation was seen in the gallbladder. Therefore, the cause of the xanthogranulomatous cholangitis in this case remains unclear.

The manifestations of this case were similar to those of other causes of bile duct stricture, and must be distinguished from gallstone disease, bile duct carcinoma, and primary sclerosing cholangitis (PSC). It is not known whether XGC and gallbladder carcinoma are associated; some authors have indicated an association, and it can be difficult to differentiate XGC from gallbladder carcinoma^[5,6]. We initially suspected that the bile stricture was PSC or a submucosal lesion like a MALToma, based on the smooth stricture of the bile duct seen in the

cholangiograms. However, we diagnosed the bile duct stricture as malignant adenocarcinoma because repeated bile duct cytology was class V adenocarcinoma. Two prospective studies of the bile cytology of biliary strictures have shown that the accuracy of brush cytology for cholangiocarcinoma is approximately 60%^[7,8]. In addition, Gerhards *et al* reported a false-positive diagnosis of malignancy resulted in a 15% resection rate for benign hilar strictures. They concluded that resection of a benign lesion mimicking a malignant stricture at the proximal bile duct could not be avoided^[9].

Xanthogranulomatous cholangitis is included in the differential diagnosis of benign and malignant strictures at the hepatic hilum. Precise reviews of all available findings are required to make the correct diagnosis. Finally, the surgeons must know that the bile cytologic diagnosis is sometimes falsely positive for malignancy.

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Transplantation of an eight-organ multivisceral graft in a patient with frozen abdomen after complicated Crohn's disease

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Abstract

To report an extended multivisceral transplantation (MVTx) including right kidney and ascending colon in a patient with complicated Crohn's disease (CD). A 36-year old female suffering from short bowel syndrome and frozen abdomen due to fistulizing CD after multiple abdominal operations underwent MVTx of eight organs including stomach, pancreatoduodenal complex, liver, intestine, ascending colon, right kidney, right adrenal gland, and greater omentum in November 2003. Immunosuppression consisted of alemtuzumab, tacrolimus and steroids. The patient was off parenteral nutrition by postoperative wk 3. She experienced one episode of pneumonia. The patient recovered completely and discharged 2.5 mo and was doing well 30 mo after MVTx. This is one of the very rare cases in which a complete multivisceral graft of eight abdominal organs was transplanted orthotopically.

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Key words: Multivisceral transplantation; Intestinal transplantation; Crohn's disease

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INTRODUCTION

Multivisceral transplantation (MVTx) has been attempted

since the idea of small bowel transplantation became a clinical reality. Starzl and Kraupp performed the first experimental MVTx in 1960^[1]. Starzl was also the first to perform a MVTx clinically in 1983 and 1986^[2]. Williams contributed another two MVTx patients whom he reported on in 1989^[3]. However, success rates were disappointing with two patients dying early after surgery due to uncontrollable bleeding and the other two due to lymphoma 3 and 6 mo after transplantation. Margreiter *et al*^[4] performed the first MVTx in an adult patient who left hospital free of total parenteral nutrition (TPN). Along with the slow but steady development of intestinal transplantation (ITx) since the late 1980s, the number of MVTx increased as well. Ninety-six MVTx had been reported to the Intestinal Transplant Registry before September 2001, accounting for approximately 13.6% of all intestinal transplants^[5]. Because the term MVTx is defined as transplantation of three or more visceral organs en-bloc, there is a considerable variation in number and combination of organs grafted. Additionally, different transplant techniques have been described reflecting the necessity for a tailor-made approach for each patient. Simultaneous MVTx and kidney transplantation (Ktx) have been reported episodically^[6-8], however none of them was performed in combination with parts of the colon.

In the following we report a MVTx including stomach, pancreatoduodenal complex, liver, intestine, ascending colon, right kidney, right adrenal gland, and greater omentum for a patient suffering from short bowel syndrome and frozen abdomen due to fistulizing Crohn's disease (CD) after multiple abdominal operations. The course was complicated due to secondary end-stage liver disease, decompensated renal insufficiency and sclerosing pancreatitis. This is one of the rare cases in which a complete cluster of eight abdominal organs was transplanted orthotopically. Special focus was put on the technical approach incorporating the right kidney including the right adrenal gland in a straight-forward fashion en-bloc into the multivisceral graft.

CASE REPORT

A 36-year old female patient was referred to our centre in June 2003 after continuous hospital stay over more than 1.5 years to undergo combined liver and intestinal transplantation because of very short bowel syndrome (15 cm residual jejunum) and total parenteral nutrition (TPN)-associated liver cirrhosis. Fistulizing CD was diagnosed in 1988. In November 2001 subtotal colectomy

was performed which was followed by postoperative ileus, recurrent intestinal leakages, recurrent anastomotic leakages with abdominal sepsis and multiple abdominal reoperations resulting in sequential subtotal small bowel resection. TPN was instituted in April 2002 rapidly leading to cholestatic liver disease. TPN-associated progressive liver cirrhosis with severe portal hypertension was diagnosed in autumn 2002. After a total of over 20 operations, the patient suffered from dehiscence of the abdominal wall until April 2004, when she was referred to our institution. Persistent intra-abdominal and transrectal bleeding due to intra-abdominal abscesses and portal hypertension required 8-10 units of packed red blood cells (RBC) per week. Urgent reoperation revealed extensive intra-abdominal scarring in terms of a frozen abdomen with complete obstruction of the abdominal cavity by the residual mesentery, extensive scarring of the retroperitoneum and an approximately 10 cm dehiscence of the abdominal wall. Hematomas and abscesses were drained, the bleeding situation could be stabilized and a closure of the abdominal wall was performed. However, since a surgical separation, also of the upper abdominal organs, was not possible due to extensive scarring, the indication for en-bloc MVTx was confirmed. In the following months, the patient suffered from recurrent decompensations of liver and kidney function.

After 5 mo, a blood group and body-weight matched, 13-year old donor was available, who died of brain death four days after a car accident. During donor procedure, preparation was limited to the resection of colon transversum, while the omentum was preserved at the greater curvature of stomach. Later on it was harvested en bloc together with the other grafts. Organs were perfused en bloc with 5 liters UW solution through the aorta, while small bowel perfusion was reduced after 1000 mL by occlusion of the superior mesenteric artery to avoid intestinal edema. The abdominal graft was harvested en bloc, including whole abdominal aorta and caval vein (CV) and all abdominal organs (Figure 1). In the back-table procedure the spleen was dissected and the left kidney was preserved for a different recipient. The aorta was sutured below the origin of the right renal artery in order to build a new celiac trunk as an offspring where all visceral arteries origin.

The recipient operation was started with a meticulous surgical dissection and exenteration of the abdominal cavity (Figure 2). The whole abdominal cavity and retroperitoneum were filled with massive collaterals because of severe portal hypertension which also explained the daily parastomal bleeding episodes prior to MVTx. Although a veno-venous bypass was used between left femoral vein and left axillar vein to reduce the venous blood flow in the abdomen, a total of 71 packed RBCs, 69 units of FFPs, and 5 units of platelet concentrates were transfused during the 11 h operation.

MVTx included stomach, pancreatoduodenal complex, small bowel, ascending colon, liver, right kidney, the adjacent right adrenal gland, and the greater omentum. Following vascular anastomoses were performed: Venous reconstruction was accomplished by inserting the retrohepatic caval vein including the right renal vein

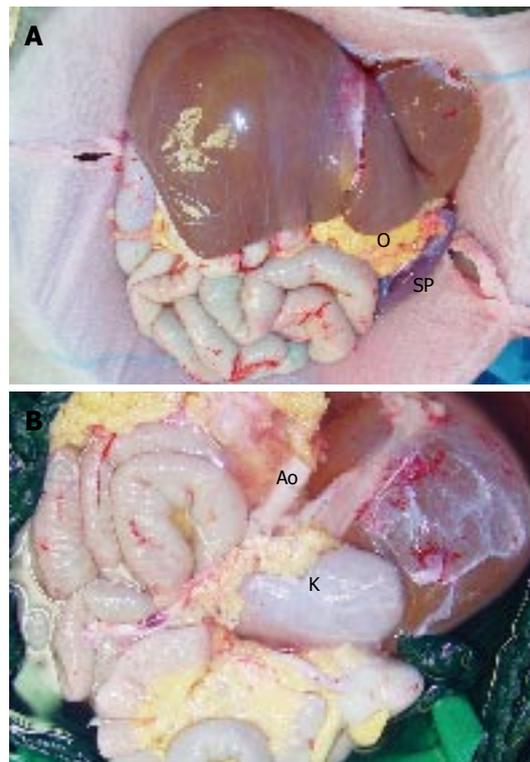


Figure 1 Graft after perfusion and removal including liver stomach, duodenal-pancreatic complex, small intestine, ascending colon and right kidney (K). The greater omentum (O) was preserved to protect the bowels in case of abdominal dehiscence. The spleen was removed at the back-table. The aorta (Ao) was sutured below the offspring of the right renal artery and used as a neo-colic trunk.

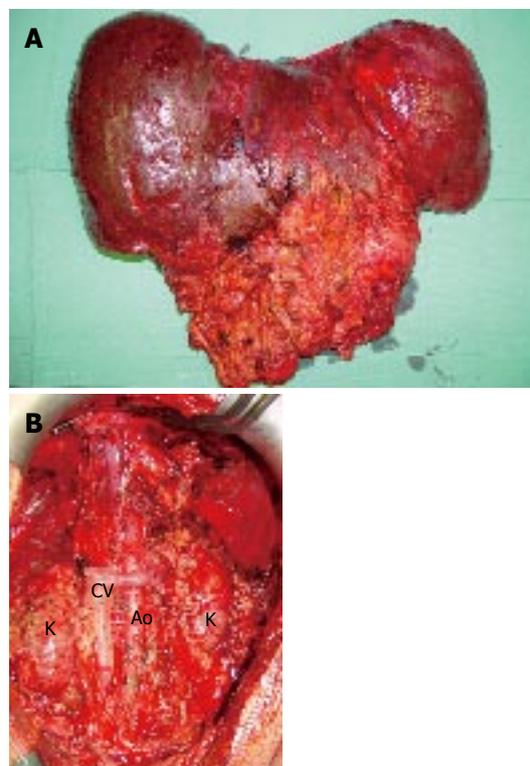


Figure 2 Recipient operation with a meticulous surgical dissection and exenteration of the abdominal cavity. **A:** Recipient's steatotic liver and reminiscence of mesenterium and duodenal-pancreatic complex; **B:** During exenteration caval vein (CV) was resected and prepared for the orthotopic placement of the multivisceral graft. The aorta (Ao) was preserved in full length and both kidneys (K) of the recipient were left in place.

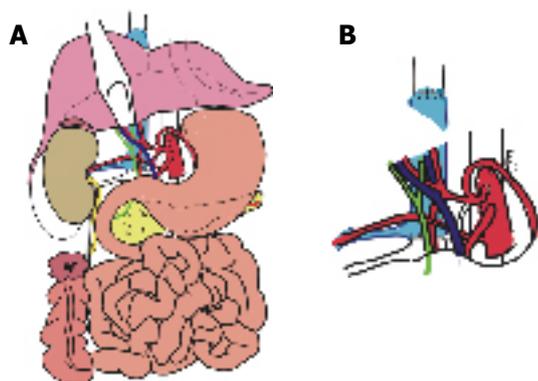


Figure 3 Venous and arterial reconstruction. **A:** Schematic demonstration of the multivisceral transplant. Venous reconstruction was accomplished by inserting the retrohepatic caval vein including the right renal vein (RV) into the recipient (CV) using standard end-to-end anastomosis of the suprahepatic inferior CV and of the infrahepatic CV between the insertion of the right donor RV and recipient RV. **B:** Arterial reconstruction was performed using the donor abdominal aorta with the natural offsprings of the celiac trunk (CT), superior mesenteric artery (SMA), and right renal artery (RA) and inserting it as aneo-celiac trunk at the site of the original recipient celiac trunk.

(RV) into the recipient (CV) using standard end-to-end anastomosis of the suprahepatic inferior CV and the infrahepatic CV between the insertion of the right donor RV and recipient RV. Arterial reconstruction was performed as previously described^[4,7,8] using the donor abdominal aorta with the natural offsprings of the celiac trunk (CT), superior mesenteric artery (SMA), and right renal artery (RA) and inserting it as neo-celiac trunk at the site of the original recipient celiac trunk (Figures 3A and 3B). After a cold ischemia time of 436 min and warm ischemia time of 31 min, reperfusion of the grafts was performed without any complications. Initial function of the organs was excellent including urine production of the transplanted kidney. Reestablishment of intestinal continuity was accomplished by end-to-side gastrostomy of the recipient cardia and donor fundus (Figure 3A), and terminal colostomy of the ascending colon in the lower right quadrant. The right donor ureter was inserted into the right recipient ureter in a side-to-end fashion. The described procedure enabled us to place the transplanted kidney and adrenal gland in almost-orthotopic position just ventrally to the remnant right recipient kidney. Because of the concomitant transplantation of the greater omentum, direct contact of the mesh with the transplanted intestine was avoided (Figure 4). The abdomen could be closed temporarily by an alloplastic mesh and approximated completely within 7 d after transplantation.

Immunosuppression consisted of alemtuzumab (Campath 1H), tacrolimus and steroids. Early enteral immunonutrition was commenced on postoperative d 1. The patient was off TPN by postoperative wk 3. She experienced one episode of pneumonia caused by citrobacter and enterococcus species which was successfully treated. The patient was discharged 2.5 mo after MVTx, with excellent graft function of all transplanted organs. During follow-up she experienced aspergillosis in the left sphenoidal sinus which was treated by drainage and antifungal therapy. There were no further complications and no rejection episodes. Tacrolimus



Figure 4 Situs after reperfusion. The right kidney (KTx) including the adrenal gland was grafted orthotopically. The greater omentum was used to cover the small bowel and the ascending colon (Co) to protect it against direct contact with the alloplastic mesh.

trough levels were adjusted to approximately 7-8 ng/mL 2 mo after MVTx was accompanied by administration of 5 mg prednisolone daily. She was doing well and recovered completely 30 mo after transplantation.

DISCUSSION

MVTx with varying combinations of visceral organs has been performed increasingly along with the improvements achieved in intestinal transplantation. Combined MVTx and KTx has been mentioned before^[9], but only documented in two patients, of them one underwent MVTx without the liver^[6,7]. Because recipients of MVTx normally have an extensive history of abdominal surgeries, abdominal exenteration and the transplant procedure itself are technically demanded. Secondary problems such as injury to adrenal glands with the potential effect of adrenal gland insufficiency may arise. We have described here a patient with previous extensive abdominal surgeries after fistulizing CD, subtotal colectomy, recurrent anastomotic leakages and prolonged intra-abdominal sepsis. Since extensive intra-abdominal and retroperitoneal scarring in terms of a frozen abdomen and retroperitoneal sclerosis were present and documented at an earlier point of time before transplantation during a massive intra-abdominal bleeding episode which required emergency surgery, the adrenal gland adjacent to the right kidney was included into the graft. This cautionary measure was proved to be important because neither of the adrenal glands was identifiable due to extensive scarring in the retroperitoneal suprarenal regions and intraoperative bleeding.

Regarding the vascular reanastomosis, two modifications of the originally described^[10] and frequently modified procedure for MVTx^[11] were used, representing the most appropriate and elegant solution for this patient. The donor abdominal aorta including CT, SMA and right RA was inserted into the recipient aorta as a neo-celiac trunk. Venous reconstruction was conducted in a way that allowed placing the right kidney and adrenal gland in almost orthotopic position with the end-to-end anastomosis between infrahepatic caval veins of the donor and recipient being located just between the right donor and recipient RAs. The piggy back procedure was not used as we were worried about potential venous congestion of the grafted organs when draining into the infrahepatic CV of the donor.

Being aware of recent reports about abdominal wall

transplantation for patients with dehiscence abdominal wall after repeat surgery^[12], we considered this option in the described patient, too. However, we decided to keep the greater omentum with the graft, thus preserving its natural role in protecting the bowels. Additionally, it induced rapid formation of granulation tissue covering the alloplastic mesh rapidly and leading to rapid reepithelialization of the abdominal wound.

In conclusion, orthotopic MVTx including the right kidney and adrenal gland is technically feasible using end-to-end cavocaval anastomoses. Transplantation of the greater omentum may obviate the need of composite grafts for reconstruction of the abdominal wall in some cases and simultaneous transplantation of the adrenal gland is effective in avoiding adrenal insufficiency due to repeat and extensive surgeries.

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Percutaneous liver biopsy complicated by hemobilia-associated acute cholecystitis

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Abstract

Liver biopsy is generally considered a safe and highly useful procedure. It is frequently performed in an outpatient setting for diagnosis and follow-up in numerous liver disorders. Since its introduction at the end of the 19th century, broad experience, new imaging techniques and special needles have significantly reduced the rate of complications associated with liver biopsy. Known complications of percutaneous biopsy of the liver include hemoperitoneum, subcapsular hematoma, hypotension, pneumothorax and sepsis. Other intra-abdominal complications are less common. Hemobilia due to arterio-biliary duct fistula has been described, which has only rarely been clinically expressed as cholecystitis or pancreatitis. We report a case of a fifteen year-old boy who developed severe acute cholecystitis twelve days after a percutaneous liver biopsy performed in an outpatient setting. The etiology was clearly demonstrated to be hemobilia-associated, and the clinical course required the performance of a laparoscopic cholecystectomy. The post operative course was uneventful and the patient was discharged home. Percutaneous liver biopsy is a safe and commonly performed procedure. However, severe complications can occasionally occur. Both medical and surgical options should be evaluated while dealing with these rare incidents.

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Key words: Hemobilia; Liver biopsy; Cholecystitis

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INTRODUCTION

The increasing clinical need for liver biopsies as well as the availability of imaging assistance have changed the nature of this procedure. A previously hazardous intervention requiring hospitalization has changed to a common, easy and safe one.

However, complications of percutaneous biopsy of the liver do occur. Hemoperitoneum, pneumothorax, sub-capsular hematoma, hypotension and sepsis have been previously documented. Hemobilia due to arterio-biliary duct fistula has been reported as well. Rarely, clinical manifestations such as cholecystitis or pancreatitis subsequently evolve.

CASE REPORT

An asymptomatic 15-year old boy presented with persistently elevated liver enzymes initially discovered on routine blood chemistries. History and physical examination revealed an athletic, healthy-appearing young man. He underwent percutaneous liver biopsy to evaluate the possible diagnosis of cryptogenic cirrhosis. The procedure was uneventful and he was discharged on the same day. The biopsy was non-diagnostic.

Twelve days later he presented to the emergency department (ED) complaining of nausea, vomiting and abdominal pain focusing in the right upper quadrant. No fever was noted and he did not appear to be jaundiced. His abdomen was soft, with mild right upper quadrant abdominal tenderness. Blood analysis showed: WBC $14.5 \times 10^3/\mu\text{L}$, total bilirubin 1.8 mg/dL, ALT/AST 54/26 U/L, amylase 60 U/L. An ultrasound (US) was performed showing a large blood clot in the gall bladder. There was no thickening of the gall bladder wall and no pericholecystic fluid evident on the US examination.

He was discharged home but returned to the ED two days later with similar complaints and physical findings. The US was repeated and the same finding was evident (Figure 1). At this time his white blood cell count was $19.5 \times 10^3/\mu\text{L}$, total bilirubin 2.6 mg/dL, ALT/AST 238/74 U/L.

Intravenous antibiotics were started, and the following day laparoscopy was performed, revealing a severely acutely inflamed gall bladder and gross evidence of macro- and micro-nodular cirrhosis. Intraoperative cholangiography (IOC) revealed a normal extrahepatic duct system and mildly dilated intrahepatic ducts (Figure 2). Laparoscopic cholecystectomy and liver biopsy were performed. The gall bladder specimen revealed severe wall-thickening,



Figure 1 Ultra-sonography of the abdomen showing distended gall bladder containing large blood clot.

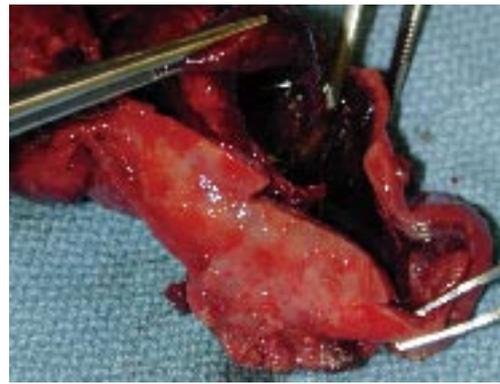


Figure 3 The opened specimen revealing a blood clot.

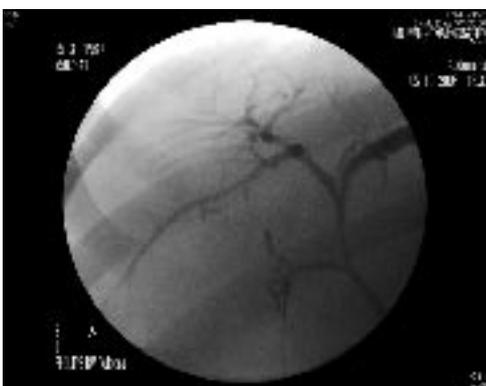


Figure 2 Intra-operative cholangiogram with normal appearing anatomy.

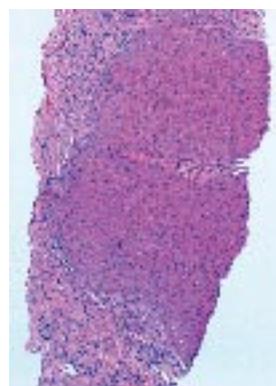


Figure 4 Hematoxylin & eosin stain of the liver sampled from the case.

and a thrombus cast of the gall bladder was found within (Figure 3). Liver biopsy revealed end stage liver disease characterized by cirrhosis and marked expansion of portal ducts (Figure 4). The patient recovered uneventfully and was discharged home on postoperative day two.

DISCUSSION

Since liver biopsy was introduced at the end of the 19th century^[1], broad experience has been gained in performing this essential procedure. Complications of percutaneous liver biopsy are fortunately uncommon, and have been well described. Ninety-six percent of such complications appear in the first 24 h^[2]. The remaining 4% usually have their clinical expression within 10 d. The incidence of complications is known to be higher in patients with either cirrhosis or neoplasm^[3]. The delay of twelve days before clinical manifestation of such a significant complication, as occurred in our patient who was ultimately found to have cirrhosis, is quite unusual.

The presence of blood in the biliary tree, hemobilia, was first described in 1948^[4]. It was first reported as a complication of liver biopsy in 1967^[5]. The incidence of hemobilia attributed to liver biopsy is less than 1%. In the largest reported series of complications induced by liver biopsies, hemobilia was noted in 4 of 68 276 patients (0.006%)^[2].

The clinical manifestations of hemobilia vary and may be subtle. When blood flows in the extra-hepatic biliary system, symptoms which are similar to those caused by gall stones might be induced. Blood clots in the gall bladder itself can mimic biliary colic or induce cholecystitis. We found only five previous cases of this in the world's literature. Clots in the common bile duct can also cause obstructive jaundice and pancreatitis similar to that caused by choledocholithiasis. In this case hemobilia caused by percutaneous liver biopsy led to the formation of a large blood clot in the gall bladder. The clinical signs of biliary colic (and subsequently acute cholecystitis) led to further evaluation and ultimately to a laparoscopic cholecystectomy.

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Comment on: Cloning and characterization of porcine aquaporin 1 water channel expressed extensively in the gastrointestinal system

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TO THE EDITOR

Sir, I read with great interest the recently published article in the *World Journal of Gastroenterology* by Jin and co-workers^[1] on the cloning and characterization of porcine aquaporin 1 water channel from the pig liver and studies on its expression in the porcine gastrointestinal system. The authors should be congratulated for making this important and valuable contribution to the field of aquaporin biology and porcine gastrointestinal physiology. However, there are a number of unresolved issues and controversies concerning the expression of aquaporins (especially aquaporin 1) in the gastrointestinal system that are worthy of additional comment and discussion by Jin and co-workers.

It is now well established that aquaporin (AQP) water channels are a family of membrane proteins that facilitate water movement across cell membranes in plants and animals. Thus far, at least 13 human aquaporins (AQP0-AQP12) have been identified^[2]. These proteins are selectively permeated by water (aquaporins)^[3] or water plus glycerol (aquaglyceroporins)^[4]. Aquaporins are strategically located at membrane sites in a variety of epithelial cells, most of which have well-defined transport functions; some are involved in fluid absorption, others in fluid secretion^[5]. Aquaporin 1 (AQP1) was the first member of the AQP family to be identified, initially

as a molecular component of the Colton blood group antigens in erythrocytes and subsequently cloned from kidney complementary DNA libraries and shown to possess water transporting activity^[6,7]. Although AQP1 has been found to be important in osmotic water movement across cell membranes of many epithelial and endothelial barriers, its expression in the gastrointestinal system is exclusively limited to microvascular endothelia (Figure 1) and other aquaporin isoforms are known to be expressed in the epithelial cell lining. These aquaporins include AQP3^[8-10], AQP4^[11-13], AQP5^[14,15], AQP8^[16,17], AQP9^[18] and AQP10^[19,22]. It is therefore clearly established that multiple aquaporin isoforms are present along the gastrointestinal system; some of these are classical aquaporins (i.e., AQP1, AQP4, AQP5, AQP8) and are probably mainly involved in water homeostasis^[23], whereas others are aquaglyceroporins (i.e., AQP3, AQP7, AQP9, AQP10) and are potentially involved in the facilitated movement of small uncharged organic molecules, such as glycerol^[4] as well as water. In the fluid-transporting epithelia of the kidney nephron, AQP1 is permeated by water, driven by osmotic gradients and AQP1 is abundant in the apical and basolateral membranes of renal proximal tubules and descending thin limbs and plays a key role in setting up and maintaining the counter-current multiplication system^[23,24]. Although AQP1 is known to be present in a number of extrarenal tissues, such as the ciliary body of the eye and the choroid plexus in the brain^[6,23,25], it has not been reported in normal epithelial cells lining the gastrointestinal system^[23]. The presence of AQP1 in gastrointestinal epithelial cells has only been reported in tumors of the colon, where it has been reported to contribute to tumor angiogenesis and the formation of high interstitial fluid pressures and high vascular permeability of tumor microvessels^[26].

Jin *et al*^[1] conclude that porcine AQP1 (pAQP1) is the first porcine aquaporin to be identified by means of molecular biology techniques. This is a valid and correct statement. However, the functional data they provide is from transfected CHO cells and red blood cells. The functional data presented by Jin *et al*^[1] may not be used to support the statement that pAQP1 plays a key role in fluid transport in epithelial and endothelial structures of the pig gut. Furthermore, the immunohistochemical and Northern blot data presented by the authors do not prove that the observed differences in pAQP1 mRNA and protein abundance in porcine liver, small intestine, colon and salivary glands are due to differential and

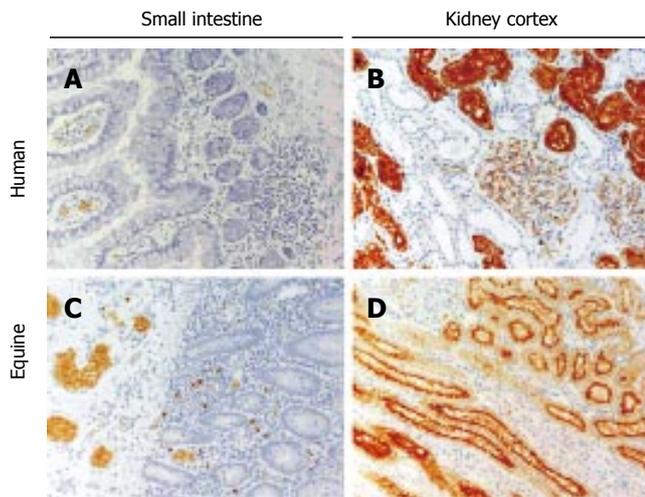


Figure 1 AQP1 expression in human and equine small intestine and kidney. In the small intestine of both species, AQP1 is present exclusively in microvessel endothelia and in erythrocytes (panels A and C). AQP1 is not expressed in epithelial cells or any other structures in the small intestine (panels A and C). In the kidney of both species specific AQP1 expression is abundant in the renal cortex, specifically in the apical (brush border) and basolateral membranes of proximal tubules and in microvessel endothelia (panels B and D). Some AQP1 expression is also seen within the glomerulus in podocytes (panels B and D).

functional expression; the differential expression observed is more likely to be the consequence of the dissimilar vascularization of these tissues which will indisputably affect the abundance of AQP1-positive endothelial cells.

Based on the evidence from studies by other investigators, it is probably premature to suggest that AQP1 is involved in fluid secretion, fluid absorption, digestive function and pathophysiology of the porcine gastrointestinal system^[1]. Multiple aquaporins are likely to be involved in the processes of fluid secretion and absorption in the digestive system. Aquaporins 3, 4, 8, 9 and 10 are also known to be expressed in strategic points along the gastrointestinal system^[13]. The presence of several different AQP isoforms along the gastrointestinal tract is likely to be related to the specific water-transporting functions of each of its segments. For example, salivary glands synthesize salivary amylase and mucus which are secreted along with electrolytes and water. The exocrine pancreas synthesizes proteases, lipases and amylases which are secreted with HCO_3^- and water. The liver and gallbladder are involved in bile salt synthesis, its secretion, storage and concentration, respectively. Gastric parietal cells secrete HCl into the lumen of the stomach and hence require AQP water channels for their secretory function. Finally, the small and large intestines absorb up to 8.5 L and 0.4 L of water every day (Figure 2).

Studies on AQP-knockout mice have shown that some of the observed phenotypes are quite mild, especially in the gastrointestinal system which is not surprising given that water transport across the digestive epithelial barriers seems to occur not only via aquaporin water channels but also via other transporters, co-transporters or channel systems. The mild phenotypes of AQP-knockout mice also suggest that other aquaporins participate in compensatory mechanisms resulting from the selective disruption of a particular AQP gene.

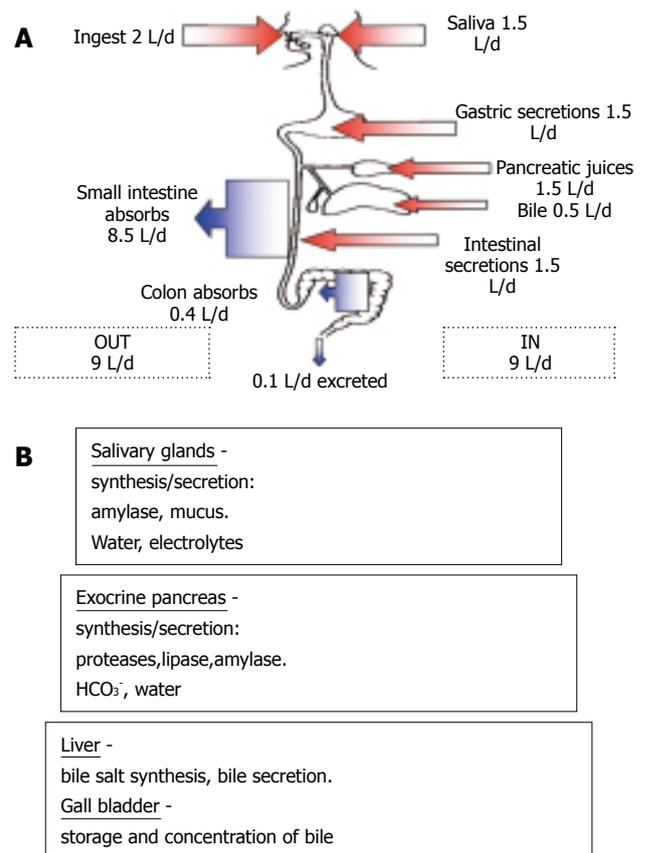


Figure 2 Summary of the gastrointestinal system's daily fluid balance (A) and the secretions of salivary glands, exocrine pancreas and liver (B).

The anatomical localization and molecular identity of AQPs in the gastrointestinal tract is not particularly well studied compared to other organs, such as the kidney. There are very few published models of water and small solute transport across the epithelium of the gut. Accordingly, the focus of future studies should be identification and subcellular localization of AQP proteins in the mammalian (human) gut. We have recently proposed a model (Figure 3) of water and small solute transport across the epithelium of the human gut which incorporates the functional contribution of AQP1, AQP3, AQP8 and AQP10^[21]. The data presented by Jin *et al*^[1] also confirm our findings^[23] and the findings of several other studies with regard to the distribution of the AQP1 protein in microvessel endothelia and bile ducts. However, further studies are still required to determine the expression of the recently identified members of the AQP family in the gastrointestinal system of humans in health and in diseases brought about by opportunistic bacteria (*i.e.*, diarrhoea, colic and endotoxemia), immune disorders of the gut, such as Crohn's disease and cancers of gastrointestinal organs. From a biological viewpoint, it would be of interest to perform comparative studies of AQP expression and function in gut tissues derived from mammals, amphibians, reptiles and fish. Also, determining the expression of AQP proteins in guts of omnivores, herbivores and carnivores will help us gain a more complete understanding of the functions of these proteins in the mammalian gastrointestinal system.

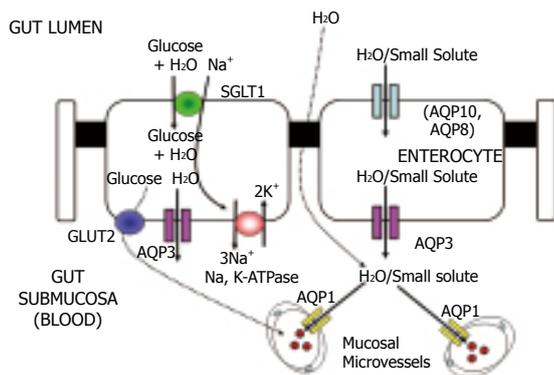


Figure 3 Model of water and small solute transport across the absorptive epithelium of the human small intestine^[21]. The potential routes for water entry into the enterocyte from the luminal (apical) side are AQP10 and the sodium/glucose co-transporter SGLT1. SGLT1 is a secondary active transporter driven by the function of basolateral Na,K-ATPase which co-transport Na⁺ and glucose along with water into enterocytes. AQP8 (shown in parentheses) has been shown to be located in the sub-apical cytoplasm of absorptive epithelial cells. The basolateral water exit pathway is most likely to be the AQP3 protein^[10,28]. Although the location of the AQP7 protein has not yet been precisely determined, it may be involved in glycerol transport in the intestinal lacteal system.

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Meetings

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- Morse SS**. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/EID/eid.htm>

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Medical treatment of cholestatic liver diseases: From pathobiology to pharmacological targets

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Abstract

Bile secretion is dependent on the coordinated functions of a number of hepatobiliary transport systems. Cholestasis may be caused by an impairment of bile secretion, an obstruction of bile flow or a combination of the two. The common consequence of all forms of cholestasis is retention of bile acids and other potentially toxic compounds in the hepatocytes leading to apoptosis or necrosis of hepatocytes and eventually to chronic cholestatic liver disease. In certain cholestatic disorders there is also leakage of bile acids into the peribiliary space causing portal inflammation and fibrosis. The following pharmacological targets for treatment of intrahepatic cholestasis can be identified: stimulation of orthograde biliary secretion and retrograde secretion of bile acids and other toxic cholephils into the systemic circulation for excretion *via* the kidneys to reduce their retention in the hepatocytes; stimulation of the metabolism of hydrophobic bile acids and other toxic compounds to more hydrophilic, less toxic metabolites; protection of injured cholangiocytes against toxic effects of bile; inhibition of apoptosis caused by elevated levels of cytotoxic bile acids; inhibition of fibrosis caused by leakage of bile acids into the peribiliary space. The clinical results of ursodeoxycholic acid therapy of primary biliary cirrhosis may be regarded as the first success of this strategy.

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Key words: Bile secretion; Biliary transport; Cholestasis; Nuclear receptors; Cholestatic liver disease; Primary biliary cirrhosis; Ursodeoxycholic acid

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INTRODUCTION

Great progress has been made in the last decade in our understanding of the molecular basis of bile formation and the pathobiology of cholestasis^[1-3]. Targets for medical therapy of cholestasis have been identified which help to understand the established treatments and facilitate the development of new drugs for cholestatic liver disease. In this short review, present concepts of bile formation and cholestasis are briefly summarized and medical treatment of cholestatic liver diseases is illustrated using primary biliary cirrhosis (PBC), the model disease for chronic cholestatic liver disease, as an example.

MOLECULAR MECHANISMS OF BILE FORMATION

Hepatocellular bile is formed by active transport of solutes into the bile canaliculi. Thereby, a local osmotic gradient is established between canalicular bile and sinusoidal plasma. This causes a flow of water, electrolytes and small solutes into the bile canaliculi, mainly *via* a paracellular pathway through the tight junctions which exhibit perm selectivity, and are impermeable for large and negatively charged solutes^[4].

The most important driving force for hepatocellular bile formation is the secretion of bile acids from the sinusoidal blood into the bile^[3]. Conjugated bile acids, which represent the major fraction of bile acids in the blood, are transported across the basolateral membrane of hepatocytes together with sodium by the sodium-taurocholate cotransporter (NTCP, *SLC10A1*). Unconjugated bile acids and a large variety of other organic anions including bilirubin are taken up by the hepatocytes *via* the organic anion-transporting polypeptide 2 (OATP2, *SLC21A6*). The rate limiting step for bile formation is the active transport of bile acids and other solutes across the canalicular membrane of hepatocytes. This concentrative step is driven by a number of ATP-dependent export pumps (ATP-binding-cassette-transport proteins also known as ABC-transporters). Bile salts are transported by the bile salt export pump (BSEP, *ABCB11*), whereas bilirubin diglucuronide, glutathione, divalent bile acids conjugates and a large variety of other conjugated organic anions are transported by the multidrug resistance associated protein 2 (MRP2, *ABCC2*)^[2].

A special ABC-transporter, namely the multidrug resistance P-glycoprotein 3 (MDR3, *ABCB4*), flips

phospholipids from the inner to the outer leaflet of the canalicular membrane. This flippase provides phosphatidylcholine for bile which forms mixed micelles with bile acids and cholesterol^[2].

The formation and final composition of bile depends on additional transporters in the canalicular membrane of hepatocytes as well as transporters in cholangiocytes which add cholangiocellular bile to hepatocellular bile. Among those, the chloride-bicarbonate anion exchanger 2 (AE2, *SLC10A2*) is present in the apical membrane of both hepatocytes and bile duct epithelial cells, whereas the cystic fibrosis transmembrane conductance regulator (CFTR, *ABCC7*), a chloride channel, is located in the apical membrane of bile duct epithelial cells only^[2].

The basolateral membrane of hepatocytes possesses a number of transporters which are expressed during cholestasis and transport solutes in a retrograde fashion back into the blood (see below). These are MRP4 (*ABCC4*) which transports bile acids together with glutathione^[5], MRP3 (*ABCC3*) which transports conjugated bilirubin and other organic anions^[2,6] and OST α /OST β , a heteromeric organic solute transporter which transports bile acids^[7]. During cholestasis, MRP3^[8] and OST α /OST β are also upregulated in the basolateral membrane of cholangiocytes^[9].

MOLECULAR MECHANISMS OF CHOLESTASIS

Cholestasis can be defined as an impairment of bile flow. The consequences are retention of bile acids, bilirubin and other cholephils in the liver and blood and a deficiency of bile acids in the intestine. Various forms of cholestasis can be caused by an impairment of bile secretion, an obstruction of bile flow or a combination of the two (Figure 1).

Impairment of bile secretion can be inborn, for instance in different forms of progressive familial intrahepatic cholestasis (PFIC), benign recurrent intrahepatic cholestasis (BRIC), or cystic fibrosis, and it also can be acquired by inflammation, toxins, drugs or hormones^[8,10].

Inborn defects of bile secretion: If BSEP is defective because of a gene mutation, PFIC2 or BRIC2^[11] can occur. PFIC2 can be identified by immunostaining of BSEP in liver biopsies^[12].

Mutations of MRP2 cause the Dubin Johnson syndrome, which is not a complete cholestasis, but a more selective defect of biliary secretion of organic anions such as bilirubin glucuronide. Mutations of MDR3 cause PFIC3 and mutations of CFTR cause cystic fibrosis^[2,8].

Acquired impairment of bile secretion: In inflammatory disorders such as sepsis, bacterial infections, viral hepatitis as well as toxin or drug-induced hepatitis, inflammatory cytokines can impair bile secretion. Thus, TNF α and IL-1 β down regulate NTCP and BSEP which are responsible for bile acid transport, as well as OATP2 and MRP2 which are responsible for transport of bilirubin and a variety of other organic ions^[13,14].

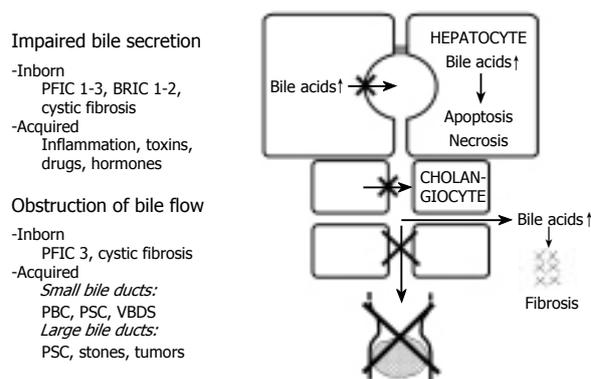


Figure 1 Causes of cholestasis. PBC: Primary biliary cirrhosis; PFIC: Progressive familial intrahepatic cholestasis; PSC: Primary sclerosing cholangitis; VBDS: Vanishing bile duct syndrome. For details see text.

Drugs can cause cholestasis by inhibiting the function of hepatobiliary transport proteins. Some drugs are known to inhibit BSEP directly from the inside of hepatocytes, which is called cis-inhibition. Examples are cyclosporine A, glibenclamide, troglitazone and bosentan^[15,16]. Other drugs, such as estradiol 17 β -D-glucuronide, must first be transported into the canalicular lumen by MRP2 and then act on BSEP from the luminal side, which is called trans-inhibition^[17].

Obstruction of bile flow can also be caused by inborn disorders, e.g. in cystic fibrosis or in PFIC3, and it can be acquired for instance in PBC, primary sclerosing cholangitis (PSC) or the vanishing bile duct syndrome (VBDS). Much more often obstructive cholestasis is caused by stones or tumours.

In cholestatic disorders caused by an initial injury of cholangiocytes (e.g. an immunological injury in the case of PBC), hydrophobic bile acids in bile (in millimolar concentrations) may aggravate the bile duct lesion and contribute to the destruction and loss of bile ducts resulting in progressive obstructive cholestasis. This may be called extracellular bile acid cytotoxicity in contrast to intracellular bile acid toxicity when bile acids accumulate in hepatocytes (in micromolar concentrations). Extracellular bile acid toxicity also occurs towards normal biliary epithelium when phospholipids in bile are low, as in the inborn defect of PFIC3 or in other "low phospholipid syndromes", in low phospholipid gallstone disease^[18] or in bile acid phospholipid imbalance in bile after liver transplantation^[19].

ADAPTIVE RESPONSES TO CHOLESTASIS

In order to compensate for the loss of biliary excretory function in cholestasis and to limit hepatocellular accumulation of potentially toxic biliary constituents, adaptive responses to cholestasis occur in the liver^[6,8,14,20,21], the kidney^[20-23] and the intestine^[22,24]. In the following only the adaptive changes in the liver are discussed.

Down regulation of NTCP and OATP2 reduces the uptake of bile acids and other organic anions in cholestasis and thus protects the hepatocytes against an overload

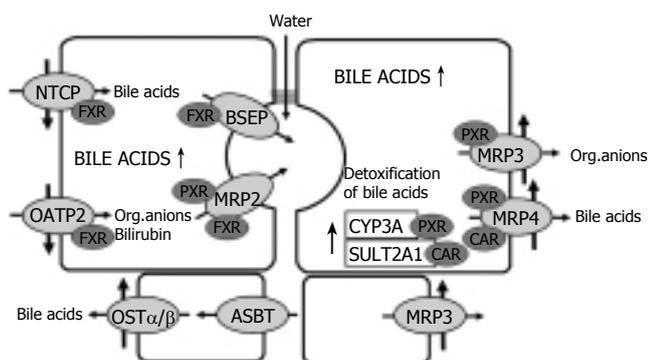


Figure 2 Adaptive responses to cholestasis. BSEP: Bile salt export pump; CAR: Constitutive androstane receptor; CYP3A: Cytochrome P450 enzyme 3A; FXR: Farnesoid X receptor; OATP: Organic anion transporting polypeptide; OST: Organic solute transporter; MRP: Multidrug resistance associated protein; NTCP: Sodium taurocholate co-transporting polypeptide; PXR: Pregnane X receptor; SULT2A1: Sulphotransferase 2A1. For details see text.

of bile acids and bilirubin^[3,25,26]. At the same time there is upregulation of MRP3 and MRP4 in the basolateral membrane^[14,22,27-29]. These transporters normally are expressed at a low level only. MRP4 pumps bile salts and bile salt conjugates together with glutathione from the cells into the blood and thus decreases bile acid retention in cholestatic hepatocytes. MRP3 mainly exports other organic anions. Prior to their extrusion from hepatocytes, hydrophobic bile acids and many xenobiotics are metabolized to more hydrophilic and less toxic compounds by cytochrome P-450(CYP) 3A enzymes. A large fraction of bile acids is sulphated by the enzyme sulfotransferase 2A1 (Figure 2).

The major players in these adaptive regulations are the nuclear receptors FXR, PXR and CAR^[30]. The farnesoid X-receptor (FXR), a bile acid sensor, is mainly involved in the down regulation of NTCP, in the maintenance of BSEP function and in the up-regulation of MRP4 and MDR3. The pregnane X receptor (PXR), to which many xenobiotics bind, is mainly responsible for the up-regulation of MRP3 and various CYP enzymes, especially the family of CYP3A enzymes. There is evidence that more than one of these nuclear receptors can act on the same transporter. Recently, it has been demonstrated that the constitutive androstane receptor (CAR) up-regulates sulfotransferase 2A1 and MRP4 in a coordinated fashion facilitating the conjugation and export of hydrophobic bile acids^[31]. In addition to PXR, the peroxisome-proliferator-activated receptor α (PPAR α) up-regulates MDR3 (Figure 2).

It is of considerable interest that besides natural bile acids, bile acid derivatives such as ethyl-chenodeoxycholic acid are ligands for FXR^[32,33]. Ligands for PXR are many xenobiotics and drugs like rifampicin. Bilirubin and phenobarbital are ligands for CAR and fibrates as well as statins (e.g. pravastatin) bind to PPAR α .

These findings open an avenue for the development of drugs which bind to nuclear receptors which enhance normal compensatory mechanisms in cholestasis for the elimination of toxic compounds *via* alternative excretory routes.

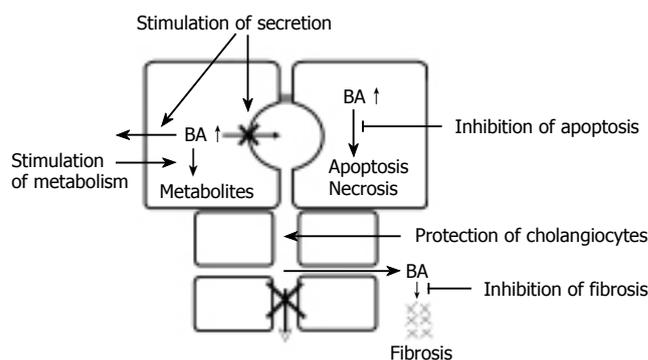


Figure 3 Targets for medical treatment of intrahepatic cholestasis. BA: Bile acids. For details see text.

TARGETS FOR PHARMACOLOGICAL THERAPY

The common consequence of all forms of cholestasis is retention of bile acids in hepatocytes. Elevated levels of bile acids then can lead to apoptosis or necrosis of hepatocytes and eventually to chronic cholestatic liver disease^[34]. In certain cholestatic disorders there is also leakage of bile acids into the peribiliary space, causing portal inflammation and fibrosis *via* induction of chemokines and cytokines^[35]. Accordingly, the following pharmacological targets for treatment of intrahepatic cholestasis can be identified (Figure 3): stimulation of orthograde biliary secretion and retrograde secretion of bile acids and other toxic cholephils into the systemic circulation for excretion by the kidneys to reduce their retention in the hepatocytes; stimulation of the metabolism of hydrophobic bile acids and other toxic compounds to more hydrophilic but less toxic metabolites; protection of injured cholangiocytes against toxic effects of bile; inhibition of apoptosis caused by elevated levels of cytotoxic bile acids; inhibition of fibrosis caused by leakage of bile acids into the peribiliary space.

Stimulation of secretion

Secretion of bile acids and other potentially toxic compounds into the bile and blood may be stimulated by enhancing transporter expression and/or function at different levels, namely the levels of transcription, translation, targeting and protein activation.

In mice both cholic acid (CA) and UDCA stimulate the expression of Bsep and Mrp2 mRNA^[36]. One must, however, be aware that these findings may not hold true for men, because considerable species differences exist with regard to binding of bile acids to nuclear receptors and regulation of transporter expression by nuclear receptors. Rifampicin, a ligand of PXR, stimulates the expression of MRP2 at the transcriptional level in man^[37].

Ursodeoxycholic acid (UDCA) stimulates targeting of the transporters Bsep and Mrp2 to the canalicular membrane in the rat *via* at least two different signalling cascades^[38-40]. Immunoelectronmicroscopy with gold particles is employed to assess localization of Bsep in the canalicular membrane and in a subapical compartment of

rat liver. Bsep and Mrp2 in the canalicular membrane are markedly reduced when taurochenodeoxycholic acid (TCGA) is administered in the perfused rat liver, but is maintained when tauroursodeoxycholic acid (TUDCA) is added^[38,40]. Enhanced expression of BSEP under UDCA treatment in men may contribute to a better elimination of bile acids from the blood. As shown by Poupon *et al*^[41], in collaboration with our group, UDCA decreases serum levels of the hydrophobic bile acid, chenodeoxycholic acid (CDCA) in PBC. As shown by Zollner *et al*^[41], expression of MRP2 mRNA and protein increases with the enrichment of UDCA in the liver during treatment of patients with PBC and UDCA. Accordingly, as shown by Poupon *et al*^[42], UDCA improves excretory function in PBC. Thus, in a randomised, placebo controlled study over two years, in patients with PBC, serum bilirubin was significantly lower in the UDCA group than in the placebo group.

Activation of transporters in the canalicular membrane by UDCA and phosphorylation may also occur^[43], which has not yet been sufficiently studied.

Stimulation of metabolism

Stimulation of the metabolism of hydrophobic bile acids produces more hydrophilic and less toxic compounds. Rifampicin, a drug used for the treatment of cholestatic pruritus, stimulates the expression of CYP3A4 mRNA in patients with gallstones. In line with this, Dilger *et al*^[44] showed that in patients with early stage PBC, rifampicin stimulates CYP3A metabolic activity as assessed by urinary 6 β -hydroxy cortisol, whereas UDCA has no effect.

Protection of cholangiocytes

Protection of cholangiocytes by making the bile more hydrophilic and less toxic appears to be an important therapeutic target. UDCA fulfils this requirement because it renders bile acid composition of bile more hydrophilic and increases biliary phospholipid secretion^[45].

Inhibition of apoptosis

Inhibition of apoptosis caused by elevated levels of hydrophobic bile acids^[46,47] may also be a therapeutic target in cholestasis. As shown by Rodrigues *et al*^[48,49], feeding of the hydrophobic bile acid deoxycholic acid (DCA) to rats increases hepatocyte apoptosis as assessed by the number of tunnel positive hepatocytes. Addition of UDCA inhibits this effect. Toxic bile acids such as CDCA can cause apoptosis of hepatocytes *via* the CD95 receptor with formation of a death inducing signalling complex (DISC) and activation of caspase 8. Caspase 8 then causes mitochondrial membrane permeability transition (MMPT) which leads to activation of effector caspases and apoptosis. In addition, UDCA stabilizes the mitochondrial membrane and inhibits MMPT and apoptosis^[48,49]. The antiapoptotic effect of UDCA has also been demonstrated in human hepatocytes^[50].

Inhibition of fibrosis

Inhibition of fibrosis may become an important

therapeutic target in the future. In the rat with common bile duct ligation, fibrosis can be inhibited by 6-ethyl CDCA (6-ECDCA). The antifibrotic effect of 6-ECDCA appears to be mediated *via* FXR and SHP^[53]. Recently, an antifibrotic effect of NOR-UDCA has been described in the Mdr2 knock-out mouse^[51]. It remains to be shown whether these findings are relevant to human cholestatic liver diseases, but they point towards a promising new way for the development of drugs to inhibit cholestatic fibrosis.

PHARMACOLOGICAL TREATMENT OF CHRONIC CHOLESTATIC LIVER DISEASES

In the following, primary biliary cirrhosis (PBC), the model disease for chronic cholestatic liver disease, is used as an example for the treatment of chronic cholestatic liver diseases by UDCA. PBC is characterized by an inflammatory lesion of interlobular bile ducts, which results in bile duct destruction and may progress to fibrosis and cirrhosis. Since the etiology of the disease is unknown, presently available therapies aim at inhibiting the underlying pathogenetic processes and delaying the progression of the disease.

The pathogenesis of this slowly progressive disease involves a still unknown immunologic injury of small interlobular bile ducts; aggravation of the bile duct lesion by cytotoxic bile acids; obstruction and loss of small bile ducts followed by cholestasis and retention of bile acids; hepatocyte injury, apoptosis, necrosis, fibrosis and eventually cirrhosis with liver failure.

UDCA, at present, is the only approved drug for PBC. It appears to exert its beneficial effects by rendering bile composition less toxic for the injured biliary epithelium, reducing the retention of bile acids in hepatocytes and inhibiting apoptosis^[10,34]. Immunosuppressive agents have met with limited success. They have been found useful in combination with UDCA in selected patients^[52,53].

In randomized, double-blind placebo-controlled trials UDCA at doses of 13-15 mg/kg body weight per day could improve serum liver tests including serum bilirubin and other serum markers of cholestasis^[42,54-56], the Mayo risk score^[56] and liver histology^[42,55]. As shown by Pares *et al*^[54] and Poupon *et al*^[57], UDCA inhibits histological progression in early stage PBC. As shown by Corpechot *et al*^[58], UDCA inhibits progression to severe liver fibrosis or cirrhosis in early stage PBC. In line with this is the observation that UDCA delays the onset of esophageal varices^[59]. A combined analysis of three of the largest trials showed that treatment with UDCA at doses of 13-15 mg/kg per day for up to 4 years can delay the time of liver transplantation or death^[60]. Within the first 2 years of treatment, however, a survival benefit was not seen. Doses lower than 10 mg/kg per day of UDCA are of little benefit in PBC^[61]. A meta-analysis of 8 randomized trials which showed no difference between UDCA and placebo in the effects on incidence of death, liver transplantation and death or liver transplantation^[62] has a number of shortcomings. In 6 of the 8 studies treatment was evaluated up to 24 mo only and the dose of UDCA was 10 mg/kg per

day or lower in two of the studies. Therefore, improvement of transplant free survival by UDCA as shown in the combined analysis of the three largest studies with doses of 13-15 mg/kg per day and a follow-up of 4 years may not have been detectable in this meta-analysis.

CONCLUSION

Better insight into the pathobiology of cholestasis has provided new concepts for pharmacological therapies of cholestatic liver diseases. Among those, therapy with UDCA has been studied most extensively. In PBC, the model disease for cholestatic liver diseases which has been highlighted in this review, the beneficial effects of UDCA have been documented by randomized controlled trials. Treatment with UDCA appears to be beneficial also in a number of other cholestatic disorders, such as primary sclerosing cholangitis (PSC)^[63-65], intrahepatic cholestasis of pregnancy^[66,67], liver disease in cystic fibrosis^[68-70], progressive familial intrahepatic cholestasis (PFIC)^[71] and some forms of drug-induced cholestasis^[10].

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EDITORIAL

Therapeutic approaches targeting intestinal microflora in inflammatory bowel disease

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Abstract

Inflammatory bowel diseases, ulcerative colitis, and Crohn's disease, are chronic intestinal disorders of unknown etiology in which in genetically susceptible individuals, the mucosal immune system shows an aberrant response towards commensal bacteria. The gastrointestinal tract has developed ingenious mechanisms to coexist with its autologous microflora, but rapidly responds to invading pathogens and then returns to homeostasis with its commensal bacteria after the pathogenic infection is cleared. In case of disruption of this tightly-regulated homeostasis, chronic intestinal inflammation may be induced. Previous studies showed that some commensal bacteria are detrimental while others have either no influence or have a protective action. In addition, each host has a genetically determined response to detrimental and protective bacterial species. These suggest that therapeutic manipulation of imbalance of microflora can influence health and disease. This review focuses on new insights into the role of commensal bacteria in gut health and disease, and presents recent findings in innate and adaptive immune interactions. Therapeutic approaches to modulate balance of intestinal microflora and their potential mechanisms of action are also discussed.

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Key words: Commensal bacteria; Prebiotics; Probiotics; Innate immunity

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INTRODUCTION

The continuous contact between commensal bacterial flora

and the single epithelial cell layer of the mucosal tissue is a characteristic feature of the gastrointestinal system. In particular, interaction between commensal bacteria and mucosal immune system plays an important role in keeping health and disease development. Inflammatory bowel diseases (IBD), ulcerative colitis (UC), and Crohn's disease (CD), are chronic intestinal disorders of unknown etiology in which in genetically susceptible individuals, the mucosal immune system shows an aberrant response towards luminal antigens such as dietary factors and/or commensal bacteria^[1]. The relation between a dysregulated bacterial ecosystem and mucosal inflammation in IBD has been demonstrated in a variety of clinical and basic literatures^[2-4]. For example, intestinal lesions of IBD predominate in the distal parts of the gastrointestinal tract where the commensal bacteria are most abundant. The presence of intestinal bacteria is essential for development of experimental colitis in several animal models, such as interleukin (IL)-10 gene knockout (KO) mice^[5], T cell receptor α -deficient mice^[6] and HLA-B27 transgenic rats^[7]. In CD, fecal stream diversion reduces gut inflammation and induces mucosal healing in the excluded intestinal segment, whereas infusion of intestinal contents rapidly induced flare-up of disease^[8].

The gastrointestinal tract has developed elaborate mechanisms to coexist with its autologous microflora, but rapidly respond to invading pathogens and then return to homeostasis with its commensal bacteria after the pathogenic infection is cleared. If these tightly regulated homeostatic mechanisms are disturbed, chronic intestinal inflammation may be induced^[9]. Previous studies demonstrated that some commensal bacteria are detrimental, and others have either no effect or have a protective action. In addition, each host has a genetically determined response to detrimental and protective bacterial species. Environmental and genetic factors modulate the relative balance of beneficial and detrimental bacterial species, suggesting that therapeutic manipulation of this balance can influence health and disease. This review focuses on new insights into the role of commensal bacteria in gut health and disease, and presents recent advances in therapeutic approaches to modulate imbalance of intestinal microflora in IBD patients.

THE INTESTINAL MICROFLORA IN HEALTH AND DISEASE

The gastrointestinal tract host a complex and dynamic microorganisms environment. Most members are from the

domain bacteria, but there are also representatives from archaea and eukarya, as well as virus^[10]. The intestinal habitat of an adult human individual contain more than 500 different species of bacteria, with 30-40 species comprising up to 99% of the total population^[10]. There is a progressive increase in the number of bacteria along the small bowel, from approximately 10^4 in the jejunum to 10^7 colony-forming units (CFU) per gram of luminal content at the ileal end, with predominance of gram-negative aerobes and some obligate anaerobes^[10]. Anaerobes are predominant in the colon, and bacterial counts reach around 10^{12} CFU per gram of luminal content. Bacteria contribute to 60% of the fecal mass. Individuals exhibit variation in the types and numbers of species within their microflora. Conventional culturing techniques could detect only ~30% of total bacteria in the gut^[11], but the use of molecular biologic techniques enhanced detection capability of numbers and diversity of microflora^[4,11].

Intestinal bacteria include native species that permanently colonize the tract and a variable set of microorganisms that transit temporarily through the gastrointestinal tract. Native bacteria are primarily acquired at birth and during the first year, but transient bacteria are being ingested continuously from the external environment. The fetal gut is sterile, and bacterial colonization, which is driven by contact between the infant and its environment, is influenced by the mode of delivery, hygiene levels and medications^[4]. At a few days after birth infant feces are rich in enterobacteria species, such as *E. coli* and *Bifidobacterium*, and these are soon influenced by feeding habits. Early colonization may also depend on genetic influences^[4]. The pattern appear to be determined in part by the host genotype, because similarity in fecal bacterial species is much higher within twins than in genetically unrelated couples who share environment and dietary habits^[10]. Intestinal microflora plays an essential role in the development of the gut immune system. Animals bred in a germ-free environment possess architectural abnormalities with crypt hyperplasia and lack of lymphoid follicle development. Immediately after exposure to microbes, the number of mucosal lymphocytes expands in the lamina propria and increases the number of IgA-secreting cells^[12,13].

Several studies using different methods have repeatedly demonstrated that the fecal microflora as well as metabolic activity differs between subjects with IBD and healthy controls^[14,15]. Recent molecular biology technique revealed that in CD patients the proportion of enterobacteria is increased^[16,17], and this finding is compatible with previous reports based in culture techniques^[14]. A large part of the dominant microflora (30%) was characterized in undefined phylogenetic groups, indicating a presence of major differences between CD and healthy individuals. Other studies confirmed that *Bacteroides vulgatus* was the only species shared by all CD patients in spite of unusual dominant species. From the results of analyses of mucosa-associated flora, Swidsinski *et al* found high concentrations of mucosal bacteria in patients with bowel inflammation, but not in controls^[18]. The concentrations of mucosal bacteria increased progressively with the severity of disease, both in inflamed and non-inflamed colon^[18]. They

Table 1 Comparison of rectal mucosal flora in UC patients

	Bacterial counts (mean \pm SD)		Positive rate (%)	
	UC	Control	UC	Control
Total	6.63 \pm 0.96	5.47 \pm 0.96		
Aerobes	6.23 \pm 0.50	5.28 \pm 2.47		
Anaerobes	6.42 \pm 1.05	5.01 \pm 0.76		
<i>Clostridium</i>	4.64 \pm 2.07	3.74 \pm 2.02	61.1	42.9
<i>Bacteroides</i>	5.33 \pm 1.71	3.96 \pm 1.66	94.4	85.7
<i>Bifidobacterium</i>	5.32 \pm 2.64	4.25 \pm 2.24	44.4	28.6
<i>Eubacterium</i>	4.73 \pm 2.13	2.73 \pm 1.53	33.3	57.1
<i>Fusobacterium</i>	0	4.14 \pm 1.95	0	28.6
<i>Actinomyces</i>	2.28 \pm 0.83	0	5.5	0
<i>Veillonella</i>	4.82 \pm 1.79	0	11.1	0
<i>Peptostreptococcus</i>	4.53 \pm 2.17	1.80 \pm 1.14	38.8	28.6
<i>Streptococcus</i>	4.93 \pm 2.33	3.03 \pm 1.47	27.7	14.3
<i>Peptococcus</i>	3.35 \pm 1.34	0	11.1	0

log₁₀ numbers of organism/g tissue. Reproduction from reference 20.

hypothesize that the healthy mucosa is capable of holding back fecal bacteria and that this function is profoundly disturbed in patients with IBD. These observations are compatible with the report by Kleessen *et al*^[19]. They demonstrated that more bacteria were detected on the mucosal surface of IBD patients than on those of non-IBD controls^[19]. Bacterial invasion of the mucosa was evident in colonic specimens from the UC patients, in the ileal and the colonic specimens from the CD patients, but no bacteria were detected in the tissues of the controls. Colonic UC specimens were colonized by a variety of organisms, such as bacteria belonging to the gamma subdivision of *Proteobacteria*, the *Enterobacteriaceae*, the *Bacteroides/Prevotella* cluster, the *Clostridium histolyticum/Clostridium lituseburense* group, the *Clostridium coccoides/Eubacterium rectale* group, high G + C Gram-positive bacteria, or sulphate-reducing bacteria, while CD samples harbored mainly bacteria belonging to the former three groups. Previously, we also reported that the bacterial counts for both aerobes and anaerobes increased in UC patients^[20]. In particular, we detected the highest bacterial counts of *Bacteroides vulgatus* (Table 1). A high agglutination titer against *B. vulgatus*, *B. fragilis*, and *C. ramosum* was detected in most UC patients, and the percentage of positive immunoreactivity was much higher in UC patients than in healthy controls. The serum immunoreactivity (IgG) against 26-kDa protein derived from *B. vulgatus* outer-membrane was much higher in UC patients (53.8%) than in the control sera (9.1%) (Figure 1). These results suggest that *B. vulgatus* and a specific antibody response directed against it may play an important role in the pathogenesis of UC^[20].

MUCOSAL RESPONSE TO LUMINAL BACTERIA

General background

The search for specific pathogens that trigger intestinal inflammation failed to produce conclusive results^[21]. Instead, it has been found that reconstitution of germ-free mice with commensal bacteria can be enough to induce IBD in several gene-deficient as well as T cell

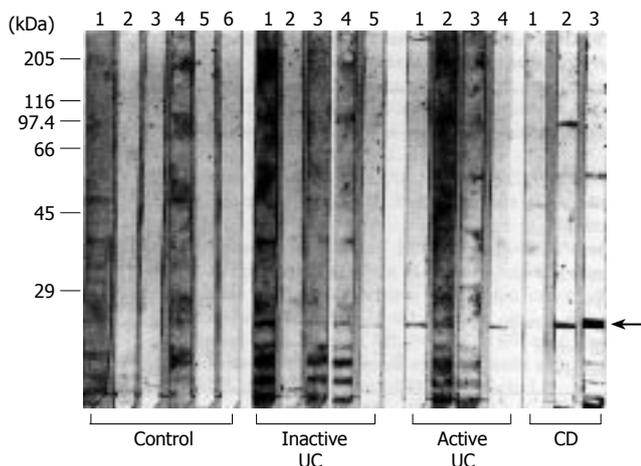


Figure 1 Western blot analysis of serum antibodies (IgG) against outer-membrane antigens of *Bacteroides vulgatus* isolated from an IBD patient. The arrow indicates specific band. Reproduced from reference 20 with permission.

transfer models of IBD in mice^[22-25]. Therefore, instead of a specific pathogen, a broad spectrum of bacteria may contribute to the induction of intestinal inflammation. Although metabolically active microbial cells and cell wall components, such as lipopolysaccharide (LPS) and peptidoglycan, present and contact to the host intestinal mucosa, pro-inflammatory responses are absent in the mucosa exposed to the resident luminal microflora. In contrast, the capability to respond to luminal pathogenic bacteria through recruitment of inflammatory cells from systemic circulation is remaining. The probable mechanisms underlying these responses are explained by the standpoints of innate and adaptive immune response^[26]. In the intestine, components of innate immunity are preexisting or rapidly activated, resulting in induction or regulation of the highly specific adaptive immune responses.

Recently, novel functions of mucosal dendritic cells (DC) have been reported. Dendritic cells are critical to innate and adaptive immunity as specialized antigen-presenting cells. Hendrik *et al* showed that lamina propria DCs form transepithelial dendrites which enable the cells to directly sample antigens, such as commensal bacterial components^[27]. It is likely that DCs take up directly intestinal antigens through transepithelial dendrites and activate an innate immune pathway that protects the mucosa from pathogenic bacteria.

Toll-like receptors and their signaling

Highly conserved structures of pathogenic and commensal bacteria, designated MAMPs (microbe-associated molecular patterns), are recognized by pattern-recognition receptors, such as TLR (Toll-like receptor)^[28-30]. TLRs comprise a family of pattern-recognition receptors that detect conserved molecular products of microorganisms, such as LPS and lipoteichoic acid (LTA), recognized by TLR4 and TLR2, respectively. TLRs are expressed both in epithelial cells and in phagocytic cells, thus functioning as sensors of microbial infection. They are critical to initiation of inflammatory and immune defense responses. The bacterial ligands recognized by TLRs are not unique

to pathogens, but are rather shared by entire classes of bacteria, and are produced by commensal bacteria as well. However, it remains unclear how the host distinguishes between pathogenic and commensal bacteria. Two major pathways are activated by TLRs^[28,30-32]. The first culminates in activating the transcription factor NF- κ B, which acts as a master switch for inflammation. It regulates the transcription of many genes that encode proteins involved in immunity and inflammation. The second leads to activation of MAP kinases p38 and Jun amino-terminal kinase (JNK), which also participate in increased transcription and regulate the stability of mRNAs that contain AU repeats. With the exception of TLR3, all TLRs activate NF- κ B and MAP kinases *via* a pathway that involves MyD88, IRAK (IL-1 receptor associated kinase)-4 and IRAK-1^[30]. There are specific differences in the ultimate gene expression profile that results from the activation of individual TLRs, although the precise mechanisms are unclear. A set of adaptor proteins that are differentially recruited to TLRs may be involved in the molecular basis of this specific gene induction^[33].

MyD88 is an adaptor protein for TLRs, and similar to TLRs, it has a Toll-IL-1 receptor (TIR) domain^[30-32,34]. Signaling may be initiated by recruitment of MyD88 to TLRs through TIR-TIR interactions. Interaction between MyD88 and TLRs leads to the recruitment of IRAK-4. IRAK-4 becomes activated and is phosphorylated to IRAK-1, resulting in activation of TRAF6^[34]. TRAF6 activation results in activation of NF- κ B and MAP kinases. Other proteins, such as Tollip, ECSIT, Pellinos, MEKK1 and MEKK3 have also been implicated in this pathway.

Recently, two different groups reported that MyD88-deficient mice reveal an increased susceptibility to dextran sodium sulfate (DSS) colitis^[35,36]. MyD88-deficient mice showed an increased mortality and morbidity, as well as aggravation of colitis, following DSS administration. In MyD88-deficient mice, mucosal proliferative zone was expanded and number of proliferating cells increased, indicating dysregulated proliferation and differentiation of intestinal epithelium. Interestingly, similar responses were also observed in TLR2- and TLR4-deficient mice. Increased susceptibility to intestinal injury in MyD88-deficient mice was accompanied with defective production of cytoprotective and reparative factors, such as IL-6, TNF, KC-1 and heat-shock proteins^[36]. These findings indicate that TLR signaling *via* the MyD88-dependent pathway conferred protection from the mortality, morbidity and colonic damage caused by the administration of the injurious agent DSS. Since DSS is capable to induce severe morbidity and mortality in wild mice where commensal microflora had been depleted by antibiotics, Authors have concluded that recognition of commensal microflora by TLRs is required for keeping intestinal homeostasis.

Several mechanisms have been proposed to explain how the epithelium discriminates pathogens from commensals in order to trigger TLR signaling. Although they express similar MAMP, pathogens differ from commensals mainly in their ability to colonize host mucosal surfaces and invade the host. Differences attributed to the differential expression of adhesion molecules. Recently-proposed speculation is that in the gut, the commensal-specific

TLR/MyD88 pathway permits the symbiotic relationship between the microflora and the host, while pathogen-specific virulence factors are required to trigger pro-inflammatory responses *via* the usage of additional TLR co-receptor and/or adaptor molecules for disease-causing organisms^[29,37].

NOD2/CARD15

The putative intracellular peptidoglycan receptor NOD2 (CARD15) is a member of the Apaf-1/CARD superfamily and is composed of an N-terminal caspase recruitment domain (CARD), a centrally located nucleotide-binding oligomerization domain (NOD) and 10C-terminal-located leucine-rich repeats (LRRs)^[38,39]. NOD2 was found to be expressed in antigen-presenting cells such as monocytes/macrophages, but more recent studies revealed abundant presence of NOD2 in epithelial Paneth cells of the small intestine as well as in other epithelial cells^[39,40]. NOD2 has been shown to recognize intracellular peptidoglycan fragments (e.g. muramyl dipeptide, MDP) through its LRR region leading to pro-inflammatory responses through activation of NF- κ B. NOD2 serves as an intracellular pattern recognition receptor to enhance host defense by inducing the production of antimicrobial peptides such as human beta-defensin-2^[39].

Several studies have shown that mutations in the LRR region of NOD2 are associated with susceptibility to Crohn's disease^[41]. The molecular mechanisms by which mutations in the NOD2 gene cause Crohn's disease are still emerging. However, it is supposed that decreased production of antimicrobial peptides, such as defensins, may promote bacterial-mediated inflammation in Crohn's disease^[39]. Recent study demonstrated that NOD2 mutation in CD potentiates NF- κ B activity and IL-1 β processing, suggesting initiation and/or promotion of mucosal inflammation^[42].

THERAPEUTIC STRATEGIES TARGETING MICROFLORA IN IBD

IBD continues to be an enigmatic disorder with obvious potential to improve therapeutic target and outcomes^[43]. Established therapies for IBD include the aminosalicylates, corticosteroids, and immunosuppressive drugs. An increasing number of novel and alternative therapeutic approaches are in progress^[43]. New biologic therapies include the targeting of proinflammatory cytokines, enhancement or infusion of anti-inflammatory cytokines, blocking intravascular adhesion molecules, and modifying T-cell functions. Recently, therapeutic approaches to modifying intestinal microflora have been attempted by using prebiotics and probiotics. In addition, antibiotic therapies continue to be used^[2,44-47].

Prebiotics

Prebiotics are nondigestible food constituents that benefit the host by selectively stimulating the growth or activity of one or a limited number of bacterial species already resident in the colon^[2]. Some examples of prebiotics are dietary fiber and some types of oligosaccharides. Intake of

prebiotics can significantly alter the colonic microflora by increasing the populations of certain bacteria and thereby quantitatively changing the composition of the microflora^[48,49]. These alterations may act beneficially, in part, by causing a luminal reduction of short-chain fatty acids (SCFAs), which are both important nutrients for the intestine and inducers of an acidic environment^[47,49-52]. Among the SCFAs, butyrate most effectively protects intestinal mucosa against injury and promotes mucosal healing^[49,53].

Lactosucrose: Lactosucrose, an indigestible oligosaccharide, is a water-soluble dietary fiber with the potency of modulating microflora. Ohkusa *et al* reported that a daily 6-gram intake of lactosucrose significantly increased the percentage of *Bifidobacterium* sp and the total number of bacteria in healthy subjects^[54,55]. The treatment also reduced fecal ammonia levels but had no effect on fecal SCFA, pH, and water content. It has been reported that an elemental diet or low-fat, low-residual diet decreases anaerobic bacteria and changes the composition of microflora in IBD patients. Teramoto *et al* showed that the continuous administration of lactosucrose for 2 wk led to an increase of *Bifidobacterium* and a decrease of *Bacteroidaceae* in patients with IBD^[55].

Oligofructose and inulin: Inulin and oligofructose are comparable to dietary fiber in that they are composed of multiple saccharide units, which are indigestible by the enzymes in mammalian small intestine. The saccharide chain in inulin is longer than in oligofructose. Inulin and oligofructose show similar physiological functions in the intestine. It is generally recognized that inulin stimulates the generation of butyrate and the growth of lactic acid bacteria (LAB) in the colons of healthy subjects^[50,56]. Videla *et al* examined the efficacy of inulin in a dextran sodium sulfate (DSS)-induced colitis model, demonstrating that it significantly attenuates inflammation as assessed by mucosal damage and both colonic eicosanoid and myeloperoxidase concentrations^[50]. The treatment also led to an increase of *Lactobacillus* and a decrease of luminal pH and fecal water content. To our knowledge, no clinical trials to confirm the benefit from either inulin or oligofructose have been performed.

Bran: The laxative effect of wheat bran has long been recognized. Although not potent, bran is used widely because of its harmlessness. A Swedish group examined the effect of wheat bran supplementation on the composition of fecal bile acid and microflora in juvenile patients with UC. Although the clinical activity was not described, wheat bran significantly decreased the number of *Bacteroides* and the concentration of total and unconjugated bile acid^[57,58].

Psyllium: Psyllium, also called Ispaghula husk or *Plantago ovata*, is a water-soluble dietary fiber. Buhman *et al* suggested that psyllium has a hypocholesterolemic effect, based on its hydrocolloid, a gel-forming potency^[59,60]. Feeding of psyllium significantly decreases the cholesterol content as well as the cholesterol 7- α hydroxylase activity in the rat liver. Hallert *et al* published the first report describing the clinical efficacy of psyllium in patients with UC^[61]. Psyllium significantly attenuates clinical symptoms compared with placebo treatment. After this first report was published, a large-scale clinical trial was organized in UC patients by Fernandez-Banares *et al*^[62]. In this trial, they

found no significant differences in the remission periods of patients given psyllium treatment and patients given sulfasalazine treatment, although there was an increase in fecal butyrate in the psyllium patients. The authors therefore concluded that psyllium may be as effective as sulfasalazine in maintaining remission in patients with CD.

Germinated barley foodstuff (GBF): GBF, which is derived from the aleuronic layer and scutellum fractions of germinated barley, consists mainly of dietary fiber and glutamine-rich protein^[47,48,51,52]. The fiber fraction of GBF consists mainly of low-lignified hemicellulose, which has a large water-holding capacity^[63]. GBF contains glutamine, which is another important nutrient for epithelial cells^[47,48,52,63]. In germination process, GBF obtains these two unique characteristics of being a glutamine-rich protein and having a conspicuous water holding capacity^[64]. In the intestinal lumen, the dietary fiber fraction of GBF is utilized efficiently by *Bifidobacterium* or *Lactobacillus* and converted to lactate and acetate. Coexistence of *Bifidobacterium* and *Eubacterium limosum* increases butyrate production from GBF^[65,66]. The endogenous bacterial butyrate produced from GBF is immediately absorbed by the intestinal epithelial cells and utilized by them as an efficient fuel. The water holding capacity of GBF is much higher than other representative water-insoluble dietary fibers, for example, wheat bran, cellulose powder, and sugar beet fiber.

Treatment of rat DSS colitis with GBF in a preventive mode led to a significant improvement of the clinical and pathological signs of colitis and a decrease in serum IL-8 and alpha 1-acid-glycoprotein^[64]. The improvements were associated with an induction of luminal butyrate and beneficial organisms, such as *Bifidobacterium* and *Eubacterium*. In a therapeutic mode, GBF was comparably effective against mucosal inflammation and more effective against diarrhea when compared with sulfasalazine^[64]. The anti-inflammatory action of GBF was markedly reduced by the concomitant administration of SCFA β -oxidation inhibitor (ibuprofen) and GBF, indicating a butyrate-dependent anti-inflammatory mechanism^[67]. In addition to its role as a preferential nutrient for colonocytes, the butyrate also acts as an anti-inflammatory agent by functionally inactivating nuclear factor κ B^[68]. Of the GBF constituents, the fiber fraction, but not its protein fraction, drastically mitigated mucosal damage with an increase of luminal butyrate. GBF significantly increased the number of *Eubacterium* and *Bifidobacterium*, with a concomitant decrease in luminal pH. In the HLA-B27 transgenic rat, a representative model of spontaneous colitis, GBF improves the clinical and pathological signs of colitis with an increase in luminal butyrate levels^[69].

The first trial enrolled 10 patients with mild to moderately active UC who had been unresponsive to or intolerant of standard treatment^[70]. The patients consumed 30 g of GBF 3 times daily for 4 wk in a nonrandomized, open-label fashion. At 4 wk, treatment with GBF resulted in clinical and endoscopic improvement with an increase in fecal butyrate. Despite continued treatment with standard drugs, the patients had an exacerbation of the disease within 4 wk after discontinuing GBF treatment. A subsequent multicenter trial with 28 patients conducted in the same fashion showed a similar benefit from GBF. Eighteen pa-

tients with mild to moderately active UC were divided into two groups using a random allocation protocol. The control group ($n = 7$) were given a baseline anti-inflammatory therapy, and the GBF-treated group ($n = 11$) received 20 to 30 g of GBF daily together with the baseline treatment. After 4 wk of observation, the GBF group showed a significant decrease in clinical activity index scores compared with the control group. No side effects related to GBF were observed^[48]. GBF increased fecal concentrations of *Bifidobacterium* and *Eubacterium limosum*. Twenty-one patients with moderately active UC patients were treated with 20-30 g/d of GBF for 24 wk. GBF significantly reduced clinical activity as compared to the control group^[52]. Furthermore, GBF prolonged remission in UC patients^[51].

Probiotics

In the intestinal lumen of IBD patients, balance between commensal and detrimental bacteria has been broken down with secondary harm on immune system activities. However, the changes in microbiotic composition may be transient; and implantation of exogenous bacteria will have a limited applicability^[2,44,46]. Probiotics are live microorganisms administered to alter the intestinal microflora and confer a beneficial effect on health^[2]. Potential mechanisms of probiotic action include competitive interactions, production of antimicrobial metabolites, influences on the epithelium, and immune modulation^[2,44,46]. However, such changes may be transient, and therefore the implantation of exogenous bacteria has a limited usefulness at present. Restoring the microbial balance using probiotics may be the most physiologic and non-toxic way to prevent and treat IBD.

VSL#3: IL-10 knockout mice develop colitis when they are raised under conventional facilities but not under germ-free conditions. Prior treatment of IL-10 knockout mice with antibiotics prevented the subsequent onset of colitis, suggesting that the exposure to intestinal bacteria during the neonatal period influences later disease progression. The use of VSL#3, a probiotic preparation containing three strains of *Bifidobacterium*, four strains of *Lactobacilli*, and one strain of *Streptococcus salivarius* ssp. *thermophilus* completely normalized the physiological transport function and barrier integrity and also inhibited mucosal TNF α and IFN γ production^[71]. *In vitro* studies showed that epithelial barrier function and resistance to Salmonella invasion could be enhanced by exposure to a proteinaceous soluble factor secreted by the bacteria found in the VSL#3 compound^[72]. There are increasing number of reports describing anti-inflammatory effects of VSL#3^[46].

In a clinical trial, daily administration of VSL#3 prevented relapse of chronic pouchitis after induction of remission by antibiotics. Moreover, every patients relapsed within 3 mo of stopping VSL#3^[73]. These were replicated^[74,75], and prospective study was performed^[76]. In this study, 2 of 20 patients (2%) receiving VSL#3 for one year developed pouchitis versus 40% of placebo-treated patients. Uncontrolled pilot studies have indicated that VSL#3 maintained remission of UC in 75% of patients over 12 mo^[73,77]. The results showed that this probiotic preparation could colonize the intestine, and might be useful in maintaining remission in UC. As another study, 32

ambulatory patients with active UC received open label VSL#3, 3600 billion bacteria daily in two divided doses for 6 wk. Treatment of patients with mild to moderate UC, not responding to conventional therapy, with VSL#3 resulted in a combined induction of remission/response rate of 77% with no adverse events. At least some of the bacterial species incorporated in the probiotic product reached the target site in amounts that could be detected^[78].

Nissle1917: Previous trials have examined the efficacy of a non-pathogenic strain of *Escherichia coli* originally called Nissle1917. In the first pilot study, capsules containing this strain of *E coli* were compared with a placebo for the maintenance of prednisolone-induced remission of colonic CD^[79]. After 12 wk of treatment, there was no significant difference between the Nissle and control groups. Rembacken *et al* described a single-center trial in which 116 patients with UC were randomized to receive either mesalazine or this non-pathogenic *E coli* strain^[80]. Initial responses to treatment were similar, with remission being noted in 75% and 68% of those receiving mesalazine and *E coli*, respectively. Even more impressive were the maintenance benefits; respectively, 73% and 67% of patients remained in remission for 12 mo^[80].

***Clostridium butyricum*:** Araki *et al* reported that the anti-colitis effect of Japanese microbial preparation (MIYA-IRI-588) was examined in a dextran sulfate sodium (DSS)-induced rodent colitis model^[81]. This preparation itself did not display any therapeutic effect. Another probiotic mixture (*Lactobacillus*, *Clostridium butyricum* and *Streptococcus faecalis*; Biothree: Towa Kasei Co., Ltd, Tokyo, Japan) was also evaluated in a DSS colitis model, by Fukuda *et al*^[82]. Although the benefits on colon histology were not significant, diarrhea was significantly decreased by treatment in comparison to the control group (66). Further trials in patients with IBD would be desirable.

***Bifidobacterium*-fermented milk:** The preventive effect of lactic acid bacteria (LAB - *Bifidobacterium breve*, *Bifidobacterium bifidum* and *Lactobacillus acidophilus*)-fermented milk was determined in SAMP1/Yit mice^[83]. Administration of LAB-fermented milk to mice reduced histological injury scores, compared with those in saline-treated or unfermented milk-treated mice. Treatment with LAB-fermented milk also reduced ileal tissue weight and myeloperoxidase activity. Moreover, the tissue contents of immunoglobulin such as IgG1 and IgG2a were lower in the inflammatory regions in the LAB-fermented milk-treated group than in the control group. A decreased release of Info and TNF α with an increase of IL-10 from mesenteric lymph node cells were observed in the LAB-fermented milk treated group.

***Lactococci*-secreting IL-10:** Steidler *et al* described the use of transgenic *Lactococcus lactis* capable of secreting bioactive IL-10 in both the DSS and IL-10 knockout models of colitis^[84,85]. The authors observed an inhibition of spontaneous colitis development in IL-10 knockout mice that was mediated by relatively low concentrations of the *Lactococcus*-borne cytokine. These experiments provide the basis for the use of genetically modified organisms designed for delivery of biologically relevant therapeutic molecules.

Synbiotics

A synbiotic is a combination of one or more probiotics and prebiotics^[86-88]. Prebiotics may enhance the survival of probiotic strains, as well as stimulating activity of the host's endogenous bacteria. Bengmark suggests that clinical effects vary from modest effects to significant effects as one goes from single-strain of probiotics < multistrain probiotics < or - < single-strain/single fiber synbiotics < multistrain/multifiber synbiotics^[87]. Kanamori *et al* reported that combination therapy with *Bifidobacterium breve*, *Lactobacillus casei*, and galactooligosaccharides dramatically improved the intestinal function in a girl with short bowel syndrome^[89]. Roller *et al* demonstrated that combination of oligofructose-enriched inulin combined with *Lactobacillus* and *Bifidobacterium* suppressed colon carcinogenesis by modulating functions of gut-associated lymphoid tissue^[90]. They also reported that same synbiotic formula stimulated secretion of secretory-IgA and IL-10 production in the cecum^[91]. A symbiotic preparation of *Bifidobacterium* combined with galacto-oligosaccharides protects against *Salmonella* infection in mice^[92]. Thus, synbiotics modulate mucosal immune responses and exert anti-inflammatory effects. We are not aware of any trial design to evaluate potential benefit of synbiotics in IBD patients.

Antibiotics

A few trials of antibacterial agents have been conducted in UC with controversial results. Oral vancomycin and intravenous metronidazole were not beneficial in active UC both as single or adjunctive given therapy^[93,94]. Tobramycin, a nonabsorbable, gram-negative specific antibiotic, induced a significant improvement in the short term compared to placebo in UC. However, the improvement was lost at long term follow-up. In addition, the association of tobramycin and metronidazole did not implemented the outcome in patients with severe UC treated by conventional therapy (steroids)^[95]. In a a, double-blind placebo-controlled trial run on a small sample, the use of rifaximin (a nonabsorbable, wide-spectrum antibiotic) led to a significant improvement in both clinical and endoscopic activity^[96].

A more definite role for antibiotics in UC is in the treatment of pouchitis, where conditions are favourable to bacterial overgrowth^[2]. It has been suggested that anaerobes induce pouch inflammation, but some investigators have found a relative increase of aerobic bacteria in pouchitis. In pouchitis, *Bacteroides* species are present in low numbers, whereas *E coli* numbers are increased, but not correlated with the degree of inflammation. Luminal pH is increased, enhancing proteolytic enzyme activity and mucin degradation. Current data suggest that dysbiosis of luminal organisms contributes to pouch inflammation in a susceptible host. Treatment with metronidazole, ciproxin and/or rifaximin leads to a significant decrease of the total number of the following anaerobes and aerobes in fecal samples: *Enterococci*, *Lactobacilli*, *Bifidobacteria*, and *Bacteroides*^[96].

Antibiotics are largely used in clinical practice for treating active CD and provide more satisfactory results. Controlled trials have supported this treatment^[97,98]. In a placebo-controlled trial, both low-dose (10 mg/kg per day)

and high-dose (20 mg/kg per day) metronidazole were more effective than a placebo in colonic CD. In a controlled trial to study the combination of metronidazole and ciprofloxacin versus methylprednisolone for active CD, after 12 wk there was no statistically significant difference in remission rates of the two groups. More recently, ciprofloxacin was shown to be effective as mesalazine in inducing remission in patients with active CD. Antibiotics do have a role in at least a subset of cases; but we must keep in mind that treatment with antibiotics may have some disadvantages, such as non-specific effect on the enteric flora, the possibility of inducing an antibiotic resistance, and the risk of *Clostridium difficile* superinfection.

In conclusion, the pathogenesis of IBD may be associated with imbalance in the intestinal microflora with a relative predominance of "aggressive" bacteria and paucity of "protective" organisms. Manipulation of the intestinal flora may represent a highly physiologic, nontoxic way to prevent and treat IBD. Although these strategies appear promising and may be actually useful in specific settings, more studies are needed to establish the relevance of these therapies.

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Living donor liver transplantation to patients with hepatitis C virus cirrhosis

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Abstract

Living donor liver transplantation (LDLT) is an alternative therapeutic option for patients with end-stage hepatitis C virus (HCV) cirrhosis because of the cadaveric organ shortage. HCV infection is now a leading indication for LDLT among adults worldwide, and there is a worse prognosis with HCV recurrence. The antiviral strategy after transplantation, however, is currently under debate. Recent updates on the clinical and therapeutic aspects of living donor liver transplantation for HCV are discussed in the present review.

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Key words: Hepatitis C virus; Living donor liver transplantation; Interferon; Rivabirin

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INTRODUCTION

The use of live donors for liver transplantation was initiated more than a decade ago as a solution to the cadaveric donor shortage for pediatric recipients^[1]. After the first successful case in an adult patient in 1994^[2], this procedure is now widely applied to adult recipients, especially in countries where the availability of brain-dead donors is severely restricted^[3] and also in the United States and European countries, due to a critical shortage

of cadaveric organs. Improved surgical techniques and the introduction of new immunosuppressive agents have enhanced the long-term results of living donor liver transplantation (LDLT), leading to an increased demand for liver transplantation that exceeds the number of potential donor organs. In initial experiences with adult LDLT in Japan, the most common indication was cholestatic liver disease, including primary biliary cirrhosis and primary sclerosing cholangitis in Japan. The number of LDLT patients indicated for hepatitis C virus (HCV) has recently increased rapidly.

A recent study^[4] of deceased donor liver transplantation (DDLT) reported that HCV infection was associated with a 23% increase in mortality and a 30% increase in the rate of graft failure. The poor results might be due to the recurrence of HCV disease in the graft^[5]. HCV-induced graft hepatitis and fibrosis/cirrhosis occur in 75% to 80% and 10% to 30% of recipients, respectively, at 5 years^[6,7]. Once liver cirrhosis is established, the cumulative probability of developing clinical decompensation is close to 50% after 1 year and survival after decompensation is extremely short^[8]. Cholestatic hepatitis occurs in approximately 10% of patients infected with HCV and leads to accelerated graft failure and death^[9]. One of the hottest debates is the possibility of increased severity of recurrent HCV in LDLT patients. The benefit of LDLT might be offset if the outcome of LDLT for HCV patients is worse than that of DDLT. In this review, we describe current trends and controversies in LDLT for patients with HCV. Our results for LDLT and HCV are also reported.

CURRENT STATUS OF LDLT

According to the Japan Liver Transplantation Society^[10], the number of adult patients (≥ 18 years old) is increasing annually, and has reached 300 in 2003. The most common indication for adults has been hepatocellular carcinoma ($n = 311$), followed by primary biliary cirrhosis ($n = 255$), and HCV-related cirrhosis without carcinoma ($n = 113$). The 1, 3, and 5 year survival rates of all the adult patients were 76%, 72%, and 69%, respectively. Those of HCV-positive patients were 76%, 73%, and 65%, respectively.

In the United States in 2000, there was a high level of enthusiasm for adult LDLT, with 49 centers performing at least one LDLT. Overall, in experienced centers, about a third of adults on the waiting list had a potential living donor and half of them had undergone LDLT; thus, LDLT might be applicable for up to 15% of individuals on the list^[11]. The enthusiasm was, however, quickly tempered

Table 1 Comparison between LDLT and DDLT for hepatitis C virus cirrhosis

Study	Author	Year	Institution	N		Dif ¹	Protocol biopsy	Findings
				LDLT	DDLT			
	Gaglio ^[23]	2003	Colombia U.	23	45	Yes	No	Cholestatic hepatitis in 17% of LDLT and 0% of DDLT ($P = 0.001$). No significant difference in incidence of Rec.
	Shiffman ^[28]	2003	Virginia Commonwealth U.	22	53	No	Yes	79% patient survival in LDLT and 91% in DDLT during 3 year (NS). No significant difference in inflammation score in liver specimen after 3 years
	Russo ^[29]	2004	UNOS data	279	3955	No	No	87% 1-year patient survival in both.
	Thuluvath ^[30]	2004	UNOS data	207	408	No	No	No significant difference in patient survival ($P = 0.6$).
	Van Vlierberghe ^[32]	2004	Ghent U.	17	26	No	No	Rec in 35% of LDLT and 38% of DDLT during 1 year ($P = 0.1$)
	Bozorgzadeh ^[34]	2004	Rochester U.	35	65	No	No	Rec in 77% of LDLT and 72% of DDLT during 1 year (NS), 89% patient survival in LDLT and 75% in DDLT during 39 mo (NS)

¹ Difference in short-term outcomes or severity of virus recurrence between living and deceased donor liver transplantation. Abbreviations: Rec, Virus recurrence; U, University; NS, not significant; UNOS, United Network for Organ Sharing.

by the death of a donor in 2002 in the United States^[12]. Since 2001, the number of patients who have undergone LDLT has declined^[13]. Currently less than 5% of all adult liver recipients use living donors. By July 2005, 2734 LDLT cases had been performed. There were 1761 adult patients and HCV was the most common indication. HCV is the most common indication for LDLT^[14] and the number of HCV-positive patients is stable, approximately 100 per year between 2000 and 2002.

By the end of 2003, 1743 LDLT cases were recorded in the European Liver Transplantation Registry^[15]. According to the Transplant Procurement Management^[16], the number of LDLT peaked in 2003 and has gradually decreased over recent years. LDLT accounts for approximately 5% of the total liver transplants performed in Europe. Among the 806 LDLT cases from October 1991 to December 2001^[17], the overall 5-year graft survival rate was 75%, better for children than for adults (80% *vs* 66% at 3 years). Cirrhosis secondary to HCV infection is a leading indication for LDLT among adults in Europe^[18]. The number of LDLT patients is shown in the Table 1.

INDICATIONS

In areas with low deceased donor organ availability, the indications for LDLT are similar to those for DDLT. In contrast, in Western countries, LDLT is conducted in an attempt to alleviate the shortage of donor organs and to decrease the mortality among the patients awaiting transplants. That is, a balance needs to be achieved between the candidate's liver disease severity and the adequacy of a partial graft for transplantation. The candidate's liver disease should be advanced to the extent that transplantation is justified, but the liver disease cannot be so advanced that a partial graft will not provide adequate hepatic mass.

According to Russo's report^[19] a substantial proportion of patients were United Network for Organ Sharing (UNOS) status 3 at the time of LDLT (43%). The policy at their centers prior to the implementation of a model for end stage liver disease (MELD)-based allocation was not to proceed with LDLT in patients meeting UNOS status 2A criteria. Their patient survival rate was 57% with an average stay of 23 d in the intensive care unit. In

comparison, 1-year patient survival was 82% in DDLT recipients who were UNOS status 2A at the time of transplant^[20].

The waiting list mortality increases in patients with advanced liver disease and patients with a MELD score of 25 have a 20% 3-mo mortality^[21]. In general, it is uncommon to proceed with LDLT in patients with MELD scores above 25. Thus, depending on the region of the country and the average MELD score at the time of the transplant within the area served by the organ procurement organization, LDLT might offer patients transplantation before they die waiting for a deceased donor liver. The lower MELD score limit with LDLT is more controversial and varies from center to center. Russo^[19] commented that they do not proceed with LDLT in candidates with MELD scores under 11.

LDLT AS A RISK FACTOR FOR RECURRENCE OF HCV

One study from Barcelona^[22] reported that LDLT patients ($n = 22$) had younger donors, less graft steatosis, more frequent biliary complications, and earlier and more severe acute hepatitis compared with DDLT ($n = 95$) patients. A report from Colombia University^[23] indicates that cholestatic hepatitis or severe HCV recurrence occurs more frequently in LDLT. These reports indicate that more intensive antiviral therapy might be necessary for recipients of living donor grafts.

The possible causes of HCV recurrence include HLA matching between donor and recipient. Because cellular immune reactions restricted by both HLA class I and II antigens are involved in the recognition of HCV peptides^[24], HLA matching between donor and recipient could potentially increase damage to the graft from recurrent viral infections by facilitating host recognition of viral antigens^[6]. Recently, a beneficial effect of a complete HLA-DQ mismatch was reported in 14 patients after transplantation for HCV cirrhosis^[25]. Another possible cause might be related to liver regeneration^[26], although recent data^[27] did not support this hypothesis. *In vitro*, HCV internal ribosome entry site activity and replication are higher in actively dividing cells, and it is possible that

viral translation is enhanced by factors that stimulate the regeneration of hepatocytes. Moreover, there are experimental data suggesting that liver regeneration induces low density lipoprotein receptor expression, which might facilitate HCV entrance into the hepatocytes.

In contrast, comparable data between LDLT and DDLT for HCV was recently reported^[28]. Russo and colleagues^[29] compared patient and graft survival in recipients transplanted for chronic HCV who received a living donor organ ($n = 279$) and deceased donor organ ($n = 3955$) using the UNOS liver transplant database. One-year patient survival was 87% in both groups and 2-year patient survival was 83% and 81% in the living donor group and deceased donor group ($P = 0.68$), respectively. Similar results (DDLT, $n = 480$ vs LDLT, $n = 207$) were obtained from another analysis using the UNOS data base^[30]. Analyses from the Mayo Clinic^[31] and Gent University^[32] also demonstrated no negative impacts of LDLT on the results of liver transplantation for HCV-related cirrhosis.

These data should be interpreted with caution, however, because of the important clinical distinction between LDLT and DDLT recipients. At the time of transplantation, the LDLT group recipients are far less sick than their DDLT group counterparts^[33]. The LDLT ($n = 35$) and DDLT ($n = 65$) data from a single institution, Rochester University, were examined^[34]. Patient survival, graft survival, rate of HCV recurrence, severity of HCV recurrence, graft loss from HCV, and interval for HCV recurrence in DDLT and LDLT were similar. It remains unclear, however, whether LDLT is truly disadvantageous compared to DDLT for HCV-positive patients because the number of cases or follow-up duration is not yet sufficient.

According to the data from Russo^[29], from 1999 to 2000, the 1-year patient survival in the LDLT group increased from 69% to 90% ($P = 0.04$), and 1-year graft survival increased from 63% to 79% ($P = 0.16$). In contrast, in the DDLT group, 1-year patient and graft survival did not substantially change from 1999 to 2000. As a result, 1-year survival rates became similar between the LDLT and DDLT groups in 2000. The results indicated an experience effect and learning curve on outcomes after LDLT for HCV. Therefore, the initial reports indicating poorer results of LDLT might be due to technical problems from a lack of experience. Recent data indicating similar results between LDLT and DDLT might be due to the increased experience with LDLT. The multicenter adult to adult LDLT cohort study (A2ALL) might soon provide some answers to the questions about recurrent HCV after LDLT and DDLT^[35].

MANAGEMENT OF HCV

Therapy for recurrence in DDLT

If HCV recurs earlier and more severely after LDLT, a specific strategy for preventing the detrimental effects of HCV on living donor grafts must be developed. One strategy might be aggressive treatment for HCV. Treatment of recurrent HCV disease with interferon and ribavirin after DDLT is used in some centers^[36-38]. One standard regimen includes interferon- α 2b (3 MU \times 3

per week) and ribavirin (1000 mg/d) for 6 mo. In a recent trial, polyethylene glycol-conjugated interferon therapy was used^[35,38-43], with a sustained viral response rate ranging from 13% to 47%.

Preemptive therapy for HCV after DDLT

Preemptive therapy in the early post-transplantation period with interferon either alone or in combination with ribavirin has been attempted in DDLT, although its effectiveness is controversial. In one study, HCV-positive recipients were randomized within 2 wk of transplantation to receive either interferon alone (3 MU \times 3 per wk, $n = 30$) or placebo ($n = 41$) for 1 year^[39]. Only 17 patients could complete 1 year of interferon therapy. Eight patients (27%) in the interferon group and 22 (54%) of the untreated patients had recurrent hepatitis ($P = 0.02$). Patient and graft survival at 2 years did not differ between the groups, however, and the rate of viral persistence was not affected by treatment.

In another controlled trial^[43], 24 recipients were randomized at 2 weeks post-transplantation to receive interferon (3 MU \times 3 per wk) or placebo for 6 mo. There were no differences in graft or patient survival. There were no differences between groups in the incidence of histological recurrence or its severity differed between groups. Recurrent HCV was delayed 408 d in treated patients versus 193 d in the control cohort.

In a case series by Mazzaferro^[44], 36 recipients were treated with interferon- α 2b (3 MU \times 3 per wk) and ribavirin (10 mg/kg per d). They started treatment at a median of 18 d after the operation and treatment continued for 11 mo. After a median follow-up of 52 mo, the 5-year patient survival was 88%. Serum HCV RNA clearance was obtained in 12 patients (33%). They did not require further antiviral treatment because of negative HCV RNA in serum and normal liver histology for a median of an additional 36 mo. The former two randomized trials on preemptive interferon monotherapy demonstrated minimal benefits of the drug. In contrast, Mazzaferro reported more encouraging results, although their protocol brings into question how long therapy is needed once embarking on a preemptive strategy.

Re-transplantation

The approach to retransplantation for recurrent HCV varies widely among the transplant centers of DDLT^[11]. The results after retransplantation for HCV (45% at 5 years) are poorer than that for other causes^[45] (56%, $P < 0.001$). The patients with recurrent HCV in the early timing and graft failure within the first year have poor outcomes after retransplantation. These individuals should be considered contraindicated for retransplantation. The experience of retransplantation for HCV in LDLT has not been well accumulated.

OUR EXPERIENCE

We performed preemptive therapy for LDLT patients with HCV infection^[38]. From 1996 to 2004, 67 patients underwent LDLT for HCV cirrhosis at the Tokyo University Hospital. The patients were 51 men and 16

women and their ages ranged from 23 to 63 years (median 55). The HCV genotype was 1b in 53 patients (79%). Forty-one patients (61%) had hepatocellular carcinoma. All the patients received the same immunosuppressive regimens with tacrolimus and methylprednisolone.

All the patients preemptively received antiviral therapy consisting of interferon α -2b and ribavirin, which was started approximately 1 mo after the operation. The therapy was continued for 12 mo after the first negative HCV RNA test. The standard regimen included interferon α -2b (3 MU \times 3 per wk) and ribavirin (800 mg/d) for 6 mo. The patients were then observed without the therapy for 6 mo. The therapy was continued for at least 12 mo even if the HCV RNA test remained positive.

Therapy was discontinued when there was significant leukopenia (< 1500 /mL), thrombocytopenia (< 50000 /mL) despite application of granulocyte colony stimulating factor (Gran[®], Sankyo, Co. Ltd., Tokyo, Japan), hemolytic anemia (hemoglobin < 8 g/L), renal dysfunction (serum creatinine > 20 mg/L), depressive psychological status, or general fatigue. The subjects were removed from the protocol if they did not continue the therapy for 12 mo due to adverse effects or could not start the therapy due to early death.

Blood counts and liver function tests were checked every 2 wk for the first month, and at 4 wk intervals thereafter. Serum samples were collected once a month for quantitative HCV RNA detection. Protocol liver biopsy was not performed. The log-rank test was used to compare the survival rate of the HCV-positive patients with the HCV-negative patients who underwent transplantation during the same period ($n = 168$).

A total of 28 patients were excluded from the analysis; 12 patients were removed from the protocol because of early death ($n = 9$) or because of drug cessation ($n = 3$). Another 16 patients are currently on the protocol and were therefore excluded from the analysis. Of the remaining 39 patients, 16 (16/39; 41%) obtained a sustained virologic response. The cumulative 5-year survival of the HCV-positive patients was 84%, comparable with that of patients negative for HCV ($n = 168$, 86%).

CONCLUSIONS

LDLT will remain an indispensable therapeutic tool for HCV related end stage liver disease and an alternative to DDLT. The association between LDLT and early HCV recurrence remains to be determined, although most of the recent papers suggest that live donor graft has no effect on short-term outcome or severity of virus recurrence. If living donor graft is associated with early HCV recurrence and consequently poorer graft survival, an aggressive antiviral protocol might improve the outcome of LDLT for HCV.

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REVIEW

Oncological problems in pancreatic cancer surgery

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Abstract

Despite the development of more sophisticated diagnostic techniques, pancreatic carcinoma has not yet been detected in the early stage. Surgical resection provides the only chance for cure or long-term survival. The resection rate has increased due to recent advances in surgical techniques and the application of extensive surgery. However, the postoperative prognosis has been poor due to commonly occurring liver metastasis, local recurrence and peritoneal dissemination. Recent molecular-biological studies have clarified occult metastasis, micrometastasis and systemic disease in pancreatic cancer. Several oncological problems in pancreatic cancer surgery are discussed in the present review.

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Key words: Pancreatic cancer; Extended resection; Molecular diagnosis; Micrometastasis; Adjuvant therapy

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INTRODUCTION

Over the past 30 years, the number of deaths in Japan due to pancreatic carcinoma has steadily increased from 4400 to 19000^[1] (Figure 1). It is the fifth most common cause of death due to malignant neoplasms (Figure 1). Regional pancreatectomy for carcinoma of pancreatic head region, introduced by Fortner^[2] in 1973, has impressed many

Japanese pancreatic surgeons. Consequently, the resection rate has gradually improved, but the postoperative prognosis is still poor in spite of the development of diagnostic modalities such as CT-scan, EUS, MRI and PET. In 1980, the Japan Pancreas Society (JPS) published the first edition of its "General Rules for Surgical and Pathological Studies on Cancer of the Pancreas". The fifth edition was published in 2002. The second English edition was published in 2003^[3]. The JPS also started a registration system for pancreatic carcinoma in 1981. According to the data of JPS, the 5-year survival of invasive ductal carcinoma of the pancreas after pancreatectomy is only 13.4%^[4] (Figure 2). JPS and UICC stage of invasive cancer and survival after pancreatectomy are shown in Figure 3^[4]. Comparison of survival curves according to the stage reveals that stratification is much better in the JPS classification than in UICC classification.

In 1981, we developed an antithrombogenic bypass catheter for the portal vein to decompress portal congestion or prevent hepatic ischemia caused by simultaneous resection of portal vein and hepatic artery^[5]. Since then, we have been aggressively performing extensive surgical resections including portal vein resection by the non touch isolation technique^[7,8] using this bypass method. The resection rate has been elevated and operative mortality has remarkably decreased. However, the postoperative prognosis is still poor due to high recurrence rate. The problems of surgical therapy for pancreatic cancer are discussed in this review.

ONCOLOGICAL PROBLEMS

Intrapancreatic carcinoma development

The indications for total pancreatectomy or pancreatoduodenectomy in pancreatic head cancer are one of the key problems in pancreatic cancer surgery. It is very important to know how the carcinoma has developed from the pancreatic head to the body or tail. A high incidence of development or multicentricity of the carcinoma of the pancreatic head to the body or tail has been reported^[9,10]. However, recent histopathological and immunocytochemical analysis of total pancreatectomy specimens have clarified that carcinoma development from head to body or tail is continuous^[11-13]. Therefore, intraoperative quick histopathological diagnosis combined with immunohistochemical staining using frozen section can diagnose intrapancreatic carcinoma development more precisely^[14,15].

Table 1 Comparative studies of extended versus standard operation for pancreatic cancer

Author	Yr	Results
Ishikawa <i>et al</i> ^[24]	1988	Retrospective study [standard (n = 37): 9%, 5-Y-S extended (n = 22): 28%, 5-Y-S
Mukaiya <i>et al</i> ^[25]	1998	Retrospective study 77 institutions, 501 patients: NS
Henne-Bruns <i>et al</i> ^[26]	2000	Retrospective study [standard (n = 26) extended (n = 46)] NS
Pedrazzoli <i>et al</i> ^[27]	1998	RCT [standard (n = 40) extended (n = 41)] overall survival: NS survival of node positive patients: extended > standard
Yeo <i>et al</i> ^[28]	2002	RCT [standard (n = 146) extended (n = 148)] mortality: NS, morbidity: extended > standard, survival: NS

RCT: Randomized controlled test; NS: Not significant.

Lymph node metastasis

Lymph node dissection is one of the important components in pancreatic cancer surgery. The high incidence of 56%^[16], 70.5%^[17], 73%^[18], 76%^[19], 77%^[20], and 86.4%^[21] in resected specimen of pancreatic cancer is the reason for wide dissection of lymph nodes in pancreatic cancer surgery. There are few reports about precise para-aortic lymph node metastasis. The incidence of para-aortic lymph node metastasis for pancreatic head carcinoma is reported to be 16% (7/44)^[17] and 26% (23/90), respectively^[20]. The incidence of pancreatic body and tail carcinoma is 13% (4/30)^[22] and 17% (4/27)^[21], respectively. The lymphatic flow from the pancreatic head tumor to the para-aortic lymph node via the posterior surface of the pancreatic head and around the superior mesenteric artery has been suspected^[17,18,23].

The efficacy of extended lymph node dissection in pancreatic cancer surgery has been suggested in a retrospective study^[24]. However, the efficacy of extended lymph node dissection has not been clarified in retrospective studies^[25,26] or in recent prospective randomized controlled tests for pancreatic cancer surgery (Table 1)^[27,28].

The incidence of perigastric lymph node metastasis in pancreatic cancer is relatively low^[20]. Therefore, pylorus preserving pancreatoduodenectomy (PPPD) is indicated for pancreatic head carcinoma, although its advantage over the classic Whipple operation has not been clarified^[29,30].

Vascular invasion

Portal vein resection is another problem in pancreatic cancer surgery. To prevent portal congestion in portal vein resection and hepatic ischemia in simultaneous resection of portal vein and hepatic artery, we developed a catheter-bypass procedure^[5,6] in our department in 1981 using antithrombogenic catheter, and isolated pancreatotomy combined with portal vein resection has thus been established^[8]. During the past 30 years, the operative mortality rate of pancreatoduodenectomy combined with portal vein resection has decreased, and portal vein resection in pancreatic cancer surgery has become a safe operative procedure. The reported mortality rate is 7.4% (2/27)^[31], 10% (6/63)^[32], 5% (3/58)^[33], 0% (0/31)^[34], 0/14^[35], 0/34^[36], 0/24^[37], and 3.2% (1/31)^[38]. From 1981 to 2003, 250 of 391 (63.9%) patients with pancreatic carcinoma underwent tumor resection in our

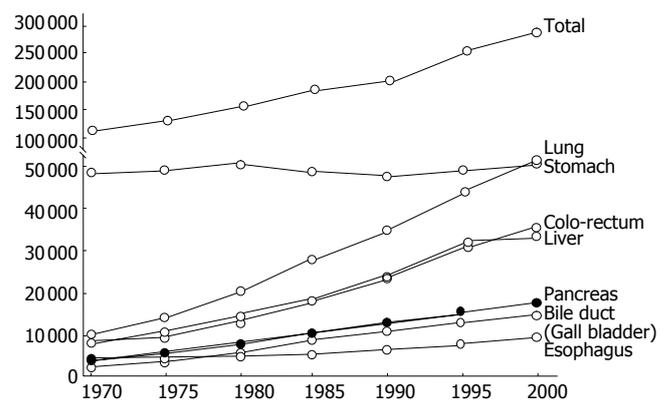


Figure 1 Trends in death due to malignant neoplasms in Japan.

department. Portal vein resection was performed in 171 of these 250 (68.4%) resected cases, and the mortality rate was 4.4% (11/250)^[39]. The indication and contraindication for portal vein resection have not yet been clarified in pancreatic cancer surgery. There are many reports about the benefit^[33,34,40] or no benefit^[41] of portal vein resection for curative resection or survival. The most important indication for portal vein resection in pancreatic cancer is the ability to obtain cancer-free surgical margins^[39].

In severe portal invasion cases, it is difficult to obtain cancer-free surgical margins, so the prognosis is poor^[39,42-44]. A recent diagnostic modality using intraportal endovascular ultrasonography provides precise information about the relationship between the pancreatic cancer and the portal vein wall, and planning of the operative procedure^[45-47].

Extrapancreatic nerve plexus invasion

Pancreatic carcinoma often invades the extrapancreatic nerve plexus^[48-51]. There is continuity of the intrapancreatic neural invasion into the extrapancreatic nerve plexus^[48]. The grade of intrapancreatic neural invasion correlates with the extrapancreatic nerve plexus invasion^[50,51] and the manner of neural invasion has no relationship with the behavior of lymph node metastasis^[50].

In pancreatic head carcinoma, complete dissection of extrapancreatic nerve plexus, especially the second portion of pancreatic head nerve plexus and nerve plexus around the superior mesenteric artery, is sometimes necessary to obtain a carcinoma-free surgical margin. However, complete resection of the nerve plexus around

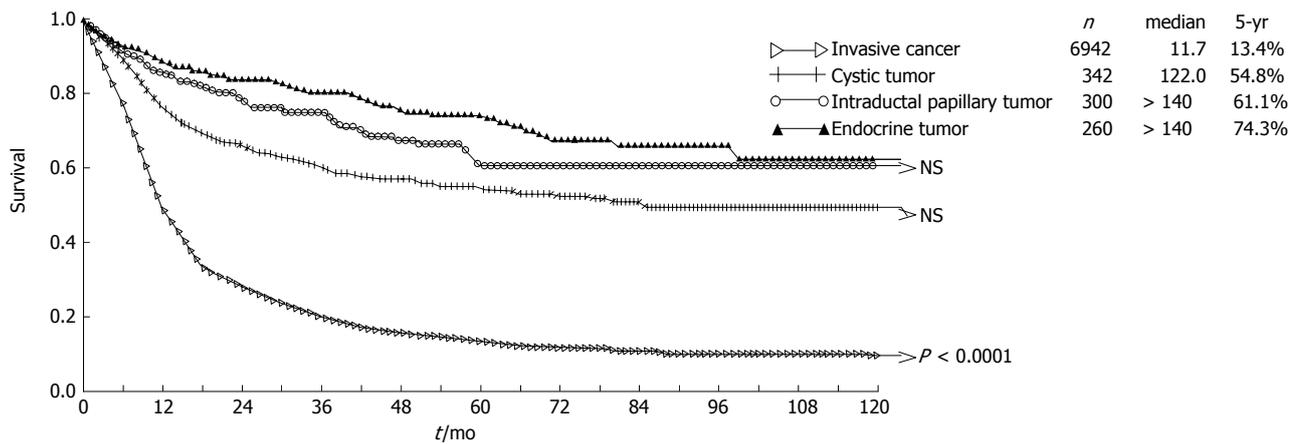


Figure 2 Histology and survival after pancreatotomy. Survival of patients who underwent pancreatotomy is shown. NS, not significant.

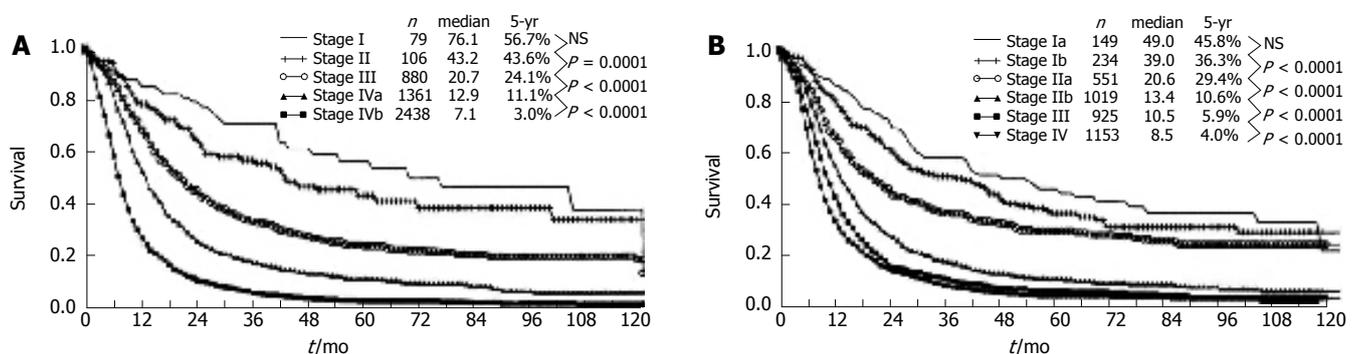


Figure 3 Survival after pancreatotomy according to JPS stage (A) and UICC stage (B). NS, not significant.

the superior mesenteric artery causes severe diarrhea after surgery, and the prognosis of positive carcinoma invasion to the extrapancreatic nerve plexus cases is very poor^[39,50,51]. The greatest cause of carcinoma-positive surgical margin is extrapancreatic nerve plexus carcinoma invasion^[39,48,50]. Recently, carcinoma invasion to the second portion of the pancreatic head nerve plexus can be diagnosed using intraportal endovascular ultrasonography^[45-47,52]. In our department, if patients have no carcinoma invasion to the second portion of the pancreatic head nerve plexus, the left semi-circular nerve plexus around the superior mesenteric artery is preserved to prevent postoperative diarrhea.

Postoperative recurrence

Even in extended surgery, a high incidence of postoperative liver metastasis, local recurrence, and peritoneal metastasis has been observed with a poor postoperative prognosis (Table 2)^[53-57]. The precise diagnosis of recurrence type is difficult even if modern diagnostic modalities are used. However, the local recurrence was 100% and the liver metastasis was 80% in 25 autopsy cases^[55]. The first cause of poor postoperative prognosis in pancreatic cancer is liver metastasis. Although occult liver metastasis may be suspected on the basis of extensive clinical data, no criteria have been definitely determined. Surgical therapy combined with effective adjuvant therapy is necessary in view of these types of recurrence.

Adjuvant therapy

Surgical therapy currently offers the only potential cure for pancreatic cancer. However the recurrence rate is very high and the long-term survival is poor.

The potential benefit of adjuvant therapy after resection of pancreatic cancer was first recognized by the randomized trial conducted by the Gastrointestinal Tumor Study Group (GITSG) using chemoradiotherapy almost 20 years ago^[58,59]. Since then, few randomized trials have shown a benefit of adjuvant treatment (Table 3)^[60-65]. The study of the European Study Group for Pancreatic Cancer (ESPAC-1) concluded that postoperative chemotherapy with fluorouracil plus leucovorin confers a benefit in terms of survival, whereas postoperative chemoradiotherapy has a deleterious effect on survival^[64]. The current study by Neuhaus *et al*^[65] indicates that the treatment with gemcitabine in patients with resected pancreatic cancer can result in improved disease-free survival as compared to observation.

A new and more effective adjuvant therapy must be established by prospective randomized trials using newly developed drugs^[66,67] or therapeutic modalities^[68]. Nevertheless, the individualized adjuvant therapy is very important in pancreatic cancer treatment^[69,70].

Occult metastasis and micrometastasis

Recent progress in immunohistochemistry and molecular biological studies has made it possible to clarify the occult metastasis and micrometastasis in pancreatic cancer. The

Table 2 Incidence of postoperative recurrence in pancreatic cancer

Author	Yr	Cases (n)	Liver (%)	Local (%)	Peritoneal (%)	Bone (%)	Lung (%)	Other (%)
Westerdahl <i>et al</i> ^[53]	1993	74	92	86.5				
Kayahara <i>et al</i> ^[54]	1993	30	60	83.3	40			
Takahashi <i>et al</i> ^[55]	1995	25	80	100	56	24	56	
Sperti <i>et al</i> ^[56]	1997	78	62	72	6			
Nakao <i>et al</i> ^[57]	1997	76	57	34	41	3	1	1

Table 3 Randomised controlled trials of adjuvant treatment for pancreatic ductal adenocarcinoma

Trial	Comparison	Adjuvant treatment	Number of patients	Conclusions
GIITSG, 1985 ^[58] , 1987 ^[59]	CRT vs OBS	2 × (20 Gy in 10 fractions + 500 mgm ⁻² 5FU d 1-3) + weekly 5FU to recurrence	49 pancreatic patients randomised	Significant increase in median survival (20 vs 11 mo, P = 0.035) in 43 eligible patients
Norway, 1993 ^[60]	CT vs OBS	AMF (40 mgm ⁻² doxorubicin, 6 mgm ⁻² mytomycin C, 500 mgm ⁻² 5FU) once every 3 wk for six courses	61 patients (47 pancreatic, 14 ampullary) randomised 46 additional nonrandomised patients	Significant increase in median survival (23 vs 11 mo, P = 0.02) in 60 pancreatic and ampullary patients combined
EORTC, 1999 ^[61]	CRT vs OBS	2 × (20 Gy in 10 fractions + 25 mgkg ⁻¹ 5FU/FA d 1-5)	218 patients (120 pancreatic, 93 ampullary) randomised	NS increase in median survival (25 vs 19 mo, P = 0.21) in 207 eligible patients NS increase in median survival in 114 eligible pancreatic patients (17 vs 13 mo, P = 0.099)
Japan, 2002 ^[62]	CT vs OBS	6 mgm ⁻² mytomycin C d 1 + 310 mgm ⁻² 5FU d 1-5 and d 15-20 followed by 100 mgm ⁻² oral 5FU daily until recurrence	508 patients (173 pancreatic, 335 bile duct/gallbladder/ampullary) randomised	Significant survival benefit in gallbladder patients No difference in 158 eligible pancreatic patients No difference in 48 eligible ampullary patients
ESPAC1, 2001 ^[63] , 2004 ^[64]	CRT vs no CRT CT vs no CT	2 × (20 Gy in 10 fractions + 500 mgm ⁻² 5FU/FA d 1-3) (20 mgm ⁻² FA + 425 mgm ⁻² 5FU d 1-5) × six cycles	289 pancreatic patients randomised	NS decrease in survival for CRT (P = 0.05) in 289 patients Significant increase in survival for CT (P = 0.009) in 289 eligible patients
CONKO-001, 2005 ^[65]	CT vs OBS	1 gm ⁻² GEM, d 1, 8, 15, every 4 wk for 6 mo	368 pancreatic patients randomised	Significant increase in median DFS (14.2 vs 7.5 mo, P < 0.05) in 356 eligible patients

CRT: Chemoradiotherapy; CT: Chemotherapy; OBS: Observation; NS: Not significant; DFS: Disease-free survival.

Table 4 Incidence of pancreatic cancer cells in peripheral blood, bone marrow, and liver tissue

Author	Yr	Incidence
Tada <i>et al</i> ^[71]	1993	Peripheral blood, K- <i>ras</i> 2/6 (33%)
Juhl <i>et al</i> ^[72]	1994	Bone marrow, immunostaining: 15/26 (58%)
Inoue <i>et al</i> ^[73]	1995	Liver tissue, K- <i>ras</i> : 13/17 (76%)
Nomoto <i>et al</i> ^[74]	1996	Peripheral blood, K- <i>ras</i> : postoperative period 10/10 (100%)
Funaki <i>et al</i> ^[75]	1996	Peripheral blood, CEAmRNA: 3/9 (33%)
Aihara <i>et al</i> ^[76]	1997	Peripheral blood, Keratin 19m RNA: 2/38 (5%)
Miyazono <i>et al</i> ^[77]	1999	Peripheral blood, CEAmRNA: 13-21 (61.9%)
Uemura <i>et al</i> ^[78]	2004	Peripheral blood, K- <i>ras</i> : 9/26 (35%)

high incidence of K-*ras* point mutation of codon 12 in pancreatic cancer has been observed. Occult pancreatic cancer cells have been detected in peripheral blood, bone marrow and liver by studies of K-*ras*, CEA mRNA, keratin 19 mRNA, along with immunocytochemical staining (Table 4)^[71-78].

Occult lymph node metastasis in pancreatic cancer has been also detected by the studies of K-*ras* and immunostaining of cytokeratin or Ber-FP4 (Table 5)^[79-83].

The incidence of cancer cells from abdominal washing cytology is shown in Table 6^[84-89]. The incidence using conventional staining is 0%-17% (Table 6)^[84,86-89]. However

Table 5 Reports of occult lymph node metastasis

Author	Yr	Results
Tian <i>et al</i> ^[79]	1992	HE: 8/56 (14%) Cytokeratin: 17/56 = (30%)
Ando <i>et al</i> ^[80]	1997	K- <i>ras</i> : paraaortic lymph nodes: 42/101 (42%)
Demeure <i>et al</i> ^[81]	1998	K- <i>ras</i> : Stage I (T1-2, N0, M0) 16/22 (73%)
Yamada <i>et al</i> ^[82]	2000	K- <i>ras</i> (-) has a better prognosis than K- <i>ras</i> (+)
Bogoevski <i>et al</i> ^[83]	2004	Ber-EP4: immunostaining 56/148 (37.8%)

Table 6 Incidence of occult peritoneal dissemination

Author	Yr	Results
Lei <i>et al</i> ^[84]	1994	Peritoneal washings, conventional cytology, 3/36 (8%), 1/11 (9%) with ascites
Juhl <i>et al</i> ^[72]	1994	Immunostaining (CEA, CA19-9,..., cytokeratin bone marrow 58%, peritoneal washings 58%)
Vogel <i>et al</i> ^[85]	1999	Peritoneal washings 39%, bone marrow 38%, one of them positive: died within 19 mo, both negative: 5 y.s. 30% (P < 0.0001)
Castillo <i>et al</i> ^[86]	1995	Laparoscopy 16/94 (17%)
Leach <i>et al</i> ^[87]	1996	4/60 (7%)
Nomoto <i>et al</i> ^[88]	1997	Conventional: 0/18 (0%), immunostaining (CEA, CA19-9): 2/18 (11%)
Nakao <i>et al</i> ^[89]	1999	Conventional: 5/66 (8%), immunostaining 14/66 (22%) prognosis between cytology positive and negative: NS

a high incidence of 58%^[72], 39%^[85], and 22%^[89] by immunocytochemical staining using monoclonal antibodies against tumor-associated antigens and cytokeratins has been reported. The difference in prognosis between positive and negative occult metastases remains controversial.

CONCLUSION

Surgical techniques for pancreatic cancer have been developed, and the resection rate has increased in Japan over the past 30 years. However, the prognosis of stage IV patients with pancreatic cancer is still poor even after aggressive surgery because of its high recurrence rate. Occult metastasis and micrometastasis have been more precisely diagnosed by immunocytochemical and molecular biological studies. On the basis of such data, adjuvant multimodal therapies targeting occult metastasis and micrometastasis with radical surgery are recommended. The effectiveness of these adjuvant multimodal therapies must be clarified and more effective adjuvant therapies must be developed.

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Diagnostic role of serum interleukin-18 in gastric cancer patients

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Abstract

AIM: To determine the current status in various aspects of gastric cancer patients and to find out the clinical correlation with prognostic role of serum interleukins in Thai patients.

METHODS: Sixty-eight patients were enrolled in this study at King Chulalongkorn Memorial Hospital during April 2003 to May 2005. Gastric cancer was histologically proven in 51 patients and gastric ulcer in 17 patients. Serum IL-6, IL-10, IL-12, and IL-18 levels were measured by enzyme-linked immunosorbent assay (ELISA).

RESULTS: There were 26 males (55.32%) and 21 females (44.68%) with their age ranging from 33 to 85 years (mean age 64.49 ± 13.83 years). The common presentations were weight loss (41.2%), dyspepsia (39.2%), and upper gastrointestinal bleeding (15.7%). A total of 35.3% gastric cancer patients and 6.3% of gastric ulcer patients were smokers ($P = 0.029$). Moreover, 32.4% of gastric cancer patients and 6.3% of gastric ulcer patients were alcoholic drinkers ($P = 0.044$). Lesion location was pyloric-antrum in 39.4%, gastric body in 39.4%, upper stomach in 12.2% and entire stomach in 6.1% of the patients. *H. pylori* infection was detected in 44.4%. The poorly-differentiated adenocarcinoma was the most common pathologic finding (60.7%). Surgical treatment was performed in 44.1% patients (total gastrectomy in 5.9%, subtotal gastrectomy in 32.4% and palliative bypass surgery in 5.9%). Systemic chemotherapy was given as an adjuvant therapy in 8.8% patients. Carcinomatosis peritonei were found in 18.8%

patients. The mean survival time was 13.03 ± 9.75 mo. The IL-18 level in gastric cancer patient group (58.54 ± 43.96 pg/mL) was significantly higher than that in gastric ulcer patient group (30.84 ± 11.18 pg/mL) ($P = 0.0001$) (95% CI was 42.20, 13.19). The cut point of IL-18 for diagnosis of gastric cancer was 40 pg/mL, the positive predictive value was 92.31%. The IL-6 level in gastric cancer patients with distant metastasis (20.21 ± 9.37 pg/mL) was significantly higher than that in those with no metastasis (10.13 ± 7.83 pg/mL) ($P = 0.037$) (95% CI was 19.51, 0.65). The role of IL-10 and IL-12 levels in gastric cancer patients was to provide data with no significant difference.

CONCLUSION: These findings demonstrate that serum IL-6 and IL-18, but not IL-10 and IL-12 levels may be the useful biological markers of clinical correlation and prognostic factor in patients with gastric cancer. Moreover, IL-18 could serve as a diagnostic marker for gastric cancer with a high positive predictive value.

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Key words: Interleukin; Gastric cancer; Diagnostic marker

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INTRODUCTION

The most recent estimates of the world-wide incidence of cancer indicate that gastric cancer is the second most frequent cancer in the world after lung cancer, with over 900 000 new cases diagnosed every year^[1].

For the risk factors, many gastric ulcer patient studies conducted all over the world have shown that the elevated risk is associated with consumption of canned fruit, pickled and smoked foods^[2,3]. Many studies have found a positive association between tobacco use and relative risk among heavy drinkers as compared to non-drinkers and gastric carcinoma^[4,5]. *H. pylori*, a Gram-negative spiral-shaped bacterium, has been established as a major etiologic

agent of chronic gastritis and peptic ulcer diseases including duodenal ulcer (DU) and gastric ulcer (GU). The role of *H pylori* infection in gastric adenocarcinoma and MALT is also increasingly recognized^[6,7].

The metastatic process of gastric cancer consists of tumor cell detachment, local invasion, motility, angiogenesis, vessel invasion, survival in the circulation, adhesion to endothelial cells, extravasation, and regrowth in different organs. In each step, causative molecules have been identified including cell-adhesion molecules, various growth factors, matrix degradation enzymes, and motility factors. Most of these molecules can be regarded as prognostic factors^[8].

Human IL-6 consists of 212 amino acids, including a hydrophobic signal sequence of 28 amino acids. Many different types of lymphoid and non-lymphoid cells produce IL-6, which is involved in the following multiple biological activities. IL-6 has a strong activity in stimulating the growth of human gastric cancer cell lines. These findings suggest that IL-6 may play a potential role in the pathogenesis of gastric cancer^[9].

IL-10 is an 18 Ku peptide and comprises 178 amino acids. This cytokine was first identified as an anti-inflammatory cytokine. The function of IL-10 is to act on the macrophages, inhibiting synthesis and suppressing gene of other cytokines^[10].

IL-12 was originally identified as a natural killer (NK) cell stimulatory factor, a disulfide-linked heterodimeric cytokine composed of 35 and 40 Ku subunits. IL-12, secreted principally by antigen presenting cells (APC), such as macrophages, B cells, and dendritic cells, activates NK cells and T cells to produce interferon- γ (IFN- γ), and augments their cytotoxic activity and proliferation. IL-12 has been recently found to induce anti-tumor effects against a variety of tumors *in vivo*^[11].

IL-18, formerly called interferon- γ -inducing factor, is a recently discovered cytokine that plays an important role in the TH1 response. IL-18 is produced by Kupffer cells, activated macrophages, keratinocytes, intestinal epithelial cells, and osteoblasts. Numerous investigations have noted the importance of IL-18 as a TH1 cytokine, especially in cooperation with IL-12, in anti-tumor immunity^[12,13].

Therefore, the purpose of this study was to investigate the possible involvement of serum IL-6, IL-10, IL-12, and IL-18 levels in determining clinicopathologic features and diagnostic yield as well as in predicting the spread of tumors, and most notably including the outcome measured as survival duration for patients with gastric carcinoma.

MATERIALS AND METHODS

Patients and blood samples

There were 68 patients enrolled in this study at King Chulalongkorn Memorial Hospital (Bangkok, Thailand) during April 2003 to May 2005. Gastric cancer was histologically proven in 51 patients and 17 gastric ulcer patients served as control. The Ethical Committee of the Faculty of Medicine, Chulalongkorn University, approved the study. Written informed consent was obtained from all patients. Immediately after blood sampling, serum was

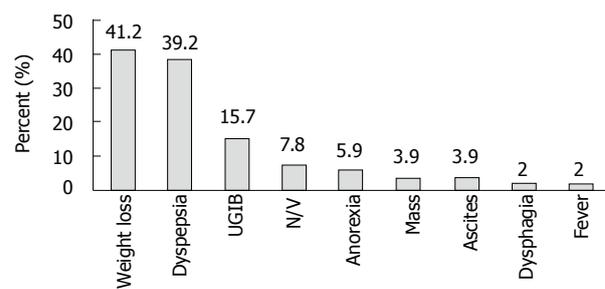


Figure 1 Symptoms of gastric cancer patients.

obtained by centrifugation at 2000 r/min for 15 min at 4°C and stored at 80°C until later analysis. Serum IL-6, IL-10, IL-12 and IL-18 levels were determined using ELISA kits (Quantikine R&D Systems, Inc., Minneapolis, MN) according to the manufacturer's instructions. Briefly, serum samples were reacted with a monoclonal antibody that recognized an epitope of rat interleukins. After 2 h incubation and washing, streptavidin peroxidase-conjugated monoclonal antibody directed to a second epitope was added. This antibody bound to the interleukins captured by the first monoclonal antibody. The color reaction was terminated by a stop solution containing sulfuric acid, and absorbance was measured at 450 nm. Results were calculated from a standard curve generated by a four-parameter logistic curve-fit and expressed in pg/mL.

The clinical data collected included age, sex, symptoms, presentation, history of cigarette smoking and alcohol drinking, lesion site, histological type, metastasis, treatment, *H pylori* status, blood chemistry, result of treatment, and survival time. The histological diagnosis was based on the morphological examination by hematoxylin and eosin-staining.

Statistical analysis

Data were expressed as mean \pm SD. Comparisons between groups were analyzed by the chi-square test for categorical variables and Student's *t* test with 95% confidence interval when appropriate for quantitative variables. Survival curves were constructed using the Kaplan-Meier method. The Pearson correlations were used. *P* values below 0.05 for a two-tailed test were considered statistically significant. All statistical analyses were performed using the SPSS software for Windows, version 11.5.

RESULTS

Of the 51 patients with gastric cancer, 26 were males (55.32%) and 21 were females (44.68%) with their age ranging from 33 to 85 years (mean age 64.49 ± 13.83 years). The common presentations included weight loss (41.2%), dyspepsia (39.2%), and upper gastrointestinal bleeding (15.7%) (Figure 1).

A total of 35.3% of gastric cancer patients and 6.3% of controls were smokers (*P* = 0.029). Moreover, 32.4% of gastric cancer patients and 6.3% of controls were alcoholic drinkers (*P* = 0.044).

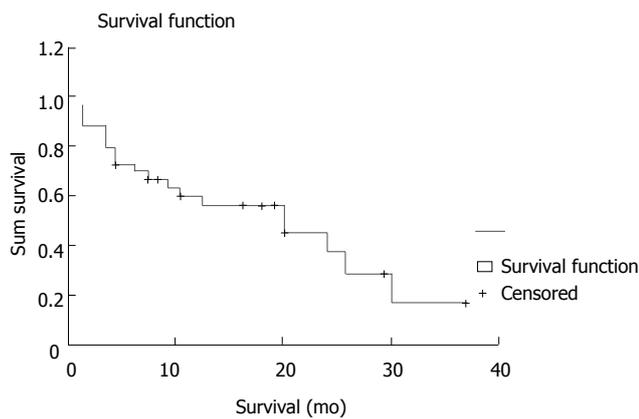


Figure 2 Mean survival time of gastric cancer patients.

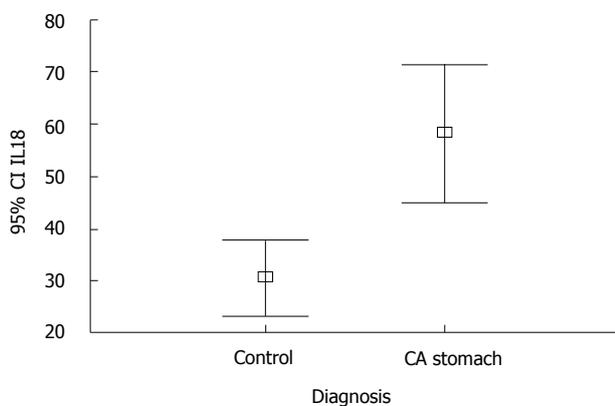


Figure 3 IL-18 levels in gastric cancer patients compared with gastric ulcer patients.

Lesion location was found in pyloric-antrum of 39.4% patients, gastric body of 39.4% patients, upper stomach of 12.2% patients and entire stomach of 6.1% patients. *H pylori* infection was detected in 44.4% patients. The poorly differentiated adenocarcinoma was the most common pathologic finding (60.7%). Surgical treatment was performed in 44.1% patients (total gastrectomy in 5.9%, subtotal gastrectomy in 32.4% and palliative bypass surgery in 5.9%). Systemic chemotherapy was given as an adjuvant therapy in 8.8% patients. Carcinomatosis peritoneii were found in 18.8% patients. The mean survival time was 13.03 ± 9.75 mo (Figure 2).

The IL-18 level in gastric cancer patient group (58.54 ± 43.96 pg/mL) was significantly higher than that in control group (30.84 ± 11.18 pg/mL) ($P = 0.0001$) (95% CI was 42.20, 13.19) (Figure 3). From the ROC curve, the cut point of IL-18 for diagnosis of gastric cancer was 40 pg/mL. The sensitivity was 52.17%, the specificity was 83.33%, and the positive predictive value was 92.31%.

The IL-6 level in gastric cancer patients with distant metastasis (20.21 ± 9.37 pg/mL) was significantly higher than that in those with no metastasis (10.13 ± 7.83 pg/mL) ($P = 0.037$) (95% CI was 19.51, 0.65) (Figure 4).

A significant clinical correlation was found between hematocrit level, serum albumin, survival time, and serum IL-6, IL-10 and IL-18 levels in gastric cancer patients (Table

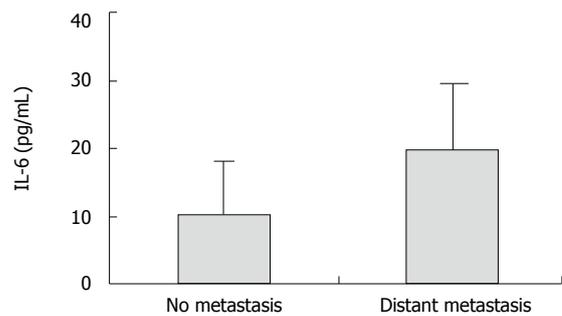


Figure 4 IL-6 levels in gastric cancer with distant metastasis compared to those with no metastasis.

Table 1 Correlation between clinical data and serum interleukins in gastric cancer

Correlations	Age	Hct	Alb	Survival (mo)	IL-6	IL-10	IL-12	IL-18
Age	1.000	0.124	-0.104	0.266	0.340	-0.135	0.122	0.109
Hct	0.124	1.000	0.593 ²	0.337 ¹	-0.466 ²	-0.096	0.163	-0.481 ²
Alb	-0.104	0.593 ²	1.000	0.473 ¹	-0.794 ²	-0.500 ²	-1.000 ²	-0.655 ²
Survival (mo)	0.266	0.337 ¹	0.473 ¹	1.000	-0.161	-0.011	0.125	-0.133
IL-6	0.034	-0.466 ²	-0.794 ²	-0.161	1.000	0.182	-0.243	0.262 ¹
IL-10	-0.135	-0.096	-0.500 ²	-0.011	0.182	1.000	-0.598	0.200
IL-12	0.122	0.163	-1.000 ²	0.125	-0.243	-0.598	1.000	-0.814
IL-18	0.109	-0.481 ²	-0.655 ²	-0.133	0.262 ¹	0.200	-0.814	1.000

^{1,2} Pearson correlation are both significant at 0.01 level (2-tailed).

1). There was no significant correlation between *H pylori* status and interleukin levels.

DISCUSSION

Gastric carcinoma is one of the most common malignant diseases worldwide. Although its incidence has declined dramatically in the United States of America and Western Europe over the past 60 years, the incidence still remains very high in developing countries^[14]. The factors leading to this variability among countries are still not clear. Some correlation exists between the occurrence of gastric carcinoma and the prevalence of *H pylori* infection in different geographical areas^[15]. Current knowledge of the detailed mechanisms underlying the interplay between biological modulators and lesions induced by *H pylori* is still incomplete. It is believed that chronic infection with *H pylori* leads to alterations of the cell cycle, including increased epithelial cell replication, increased rate of cell death (apoptosis) and production of oxidants^[16,17]. In this study, the prevalence of *H pylori* was 44.4%. There was no significant correlation between *H pylori* status and interleukin levels.

In agreement with other studies, our study examined the effect of cigarette smoking and alcohol drinking as a risk factor for gastric cancer. From the mechanism viewpoint, the direct carcinogenic effect of cigarette smoking may derive from precursor gastric lesions. Indirect effects of inhaled cigarette smoking may involve

the nitrosamines in stomach. Alcohol could act as a contributory factor by causing chronic irritation of the gastric mucosa^[4,5].

Weight loss and dyspepsia were the most frequent initial symptoms in our patients, being consistent with those in Western studies^[18,19]. The current data indicate that the proportion of early, curable gastric cancer is small among Thai patients. Most of the cases in our study were in advanced stage (stages III and IV) with a very poor 5-year survival.

IL-18 is synthesized as an inactive precursor (pro-IL-18, 24 kDa), which is cleaved by IL-1 β -converting enzyme (ICE or caspase-1) into an active 18 kDa mature form^[20,21]. IL-18 has multiple biological activities *via* its capacity of stimulating innate immunity and both Th1 and Th2-mediated responses. It also exerts anti-tumor effects that are mediated by enhancement of NK cell activity, reduction of tumorigenesis, induction of apoptosis and inhibition of angiogenesis in tumor cells^[22,23]. In addition, recent data suggest that an inappropriate production of IL-18 contributes to the pathogenesis of cancers and may influence the clinical outcome of patients^[24]. This is the first study demonstrating that the levels of serum IL-18 are significantly elevated in gastric cancer patients compared with gastric ulcer patients. Although a high serum IL-18 level was not used as a significant prognostic factor in terms of overall survival, it was used as a diagnostic factor. The cut point of IL-18 for diagnosis of gastric cancer was 40 pg/mL. The sensitivity was 52.17%, the specificity was 83.33%, the positive predictive value was 92.31%. The pathways for IL-18 production and its mechanisms of action in patients with gastric cancer remain to be determined.

IL-6 is involved in many biological activities, including T-cell and B-cell growth and differentiation, IL-2 production and IL-2 receptor expression, hematopoietic stem cell growth, megakaryocyte maturation, acute phase protein synthesis, macrophage differentiation, mesangial cell, keratinocyte, and osteoclast cell growth, hybridoma, plasmacytoma, and myeloma growth, and stimulation of cancer-cell growth^[25]. Our data show that the IL-6 level in gastric cancer patients with distant metastasis is significantly higher than that in those with no metastasis. As a proof of the mechanism of IL-6 action, Tamm *et al*^[26] showed that IL-6 makes cancer cells increase their motogenic activity by autocrine pathway. IL-6 secreted from the cancer cells combines with IL-6, which is expressed on the surface of cancer cells, IL-6 and IL-6 α act on the cancer cells directly. IL-6 may act through HGF on cancer cells by promoting and accelerating invasion as well as lymph node and/or hepatic metastasis^[27].

IL-12 is an immunoregulatory cytokine that triggers the development of a specific T cell-mediated immune response. IL-12 enhances the proliferation, cytokine production, and cytotoxic activity of T lymphocytes and NK cells, with consequent anti-tumor activity^[11]. Lissoni *et al*^[28] reported that serum IL-12 level is significantly higher in patients with metastatic renal cell carcinoma and breast cancer than in those with local solid neoplasms. Uno *et al*^[29] reported that phytohemagglutinin-induced production of IL-12 is lower in patients with gastric cancer

than in healthy controls, by examining peripheral blood mononuclear cells *in vitro*. In this study, we could not demonstrate any significant uses of IL-12 in gastric cancer patients as compared with gastric ulcer patients. Shibata *et al*^[30] reported that the production of IL-12 decreases significantly with advancing stages, and is the lowest in patients with distant metastases and cachexia.

For IL-10 level, the data indicate that intracellular IL-10 status on monocytes in patients with advanced gastric cancer is significantly increased compared with those with early disease or healthy individuals^[31]. In this study, we also found a correlation between IL-6, IL-10, and IL-18 levels and serum albumin. In our previous report, serum albumin level is another prognostic factor in gastric cancer at initial diagnosis, and the median survival time is reduced with a decrease in serum albumin level^[32].

In conclusion, our data demonstrate that serum IL-6 and IL-18, but not IL-10 and IL-12 levels, may be the useful biological markers for clinical correlation and prognostic factor in patients with gastric cancer. Moreover, IL-18 could serve as a diagnostic marker for gastric cancer with a high positive predictive value. Thus, the detailed mechanisms of IL-6 and IL-18 involving tumor progression should be further investigated.

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LIVER CANCER

Hot water-extracted *Lycium barbarum* and *Rehmannia glutinosa* inhibit proliferation and induce apoptosis of hepatocellular carcinoma cells

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LBE-treated (2, 5 g/L) groups, and 110% and 132% of the control group compared with the RGE -treated (5, 10 g/L) groups after 24 h.

CONCLUSION: Hot water-extracted crude LBE (2-5 g/L) and RGE (5-10 g/L) inhibit proliferation and stimulate p53-mediated apoptosis in HCC cells.

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Key words: *Lycium barbarum* extract; *Rehmannia glutinosa* extract; Proliferation; Apoptosis; Hepatocellular carcinoma

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Abstract

AIM: To investigate the effect of hot water-extracted *Lycium barbarum* (LBE) and *Rehmannia glutinosa* (RGE) on cell proliferation and apoptosis in rat and/or human hepatocellular carcinoma (HCC) cells.

METHODS: Rat (H-4-II-E) and human HCC (HA22T/VGH) cell lines were incubated with various concentrations (0-10 g/L) of hot water-extracted LBE and RGE. After 6-24 h incubation, cell proliferation ($n = 6$) was measured by a colorimetric method. The apoptotic cells ($n = 6$) were detected by flow cytometry. The expression of p53 protein ($n = 3$) was determined by SDS-PAGE and Western blotting.

RESULTS: Crude LBE (2-5 g/L) and RGE (2-10 g/L) dose-dependently inhibited proliferation of H-4-II-E cells by 11% ($P < 0.05$) to 85% ($P < 0.01$) after 6-24 h treatment. Crude LBE at a dose of 5 g/L suppressed cell proliferation of H-4-II-E cells more effectively than crude RGE after 6-24 h incubation ($P < 0.01$). Crude LBE (2-10 g/L) and RGE (2-5 g/L) also dose-dependently inhibited proliferation of HA22T/VGH cells by 14%-43% ($P < 0.01$) after 24 h. Crude LBE at a dose of 10 g/L inhibited the proliferation of HA22T/VGH cells more effectively than crude RGE ($56.8\% \pm 1.6\%$ vs $70.3\% \pm 3.1\%$ of control, $P = 0.0003 < 0.01$). The apoptotic cells significantly increased in H-4-II-E cells after 24 h treatment with higher doses of crude LBE (2-5 g/L) and RGE (5-10 g/L) ($P < 0.01$). The expression of p53 protein in H-4-II-E cells was 119% and 143% of the control group compared with the

INTRODUCTION

According to the official report by the Department of Health, Taiwan, malignant tumor is the first leading cause of death in 2004. Among cancers, hepatocellular carcinoma (HCC) is the second leading cause of death. The mortality rate for HCC is 31.17 per 100 000, accounting for 17.9% of cancer deaths in 2004. The rising incidence of HCC in at-high-risk patients with chronic hepatitis B or C is an important issue in Taiwan. Although early diagnosis and treatment improve survival, HCC is rarely cured and recurs frequently after regional therapy or transplantation^[1]. Recently, preventing HCC formation and HCC therapy are major research focuses.

Both *Lycium barbarum* (LBE) and *Rehmannia glutinosa* (RGE) have been commonly used as traditional Chinese medicine and herbal foods for health promotion in China. The active components of the fruit of LBE and the dried root of RGE primarily contain water-soluble polysaccharides. LBE and RGE can be extracted with hot water followed by precipitation with ethanol to obtain high quantity of polysaccharides^[2-4]. Polysaccharide-containing active components purified from these herbs have been recently studied for their physiological and pharmaceutical activities. LBE polysaccharides as glycopeptides isolated

from the fruit of LBE^[5,6] are water soluble and potent in immunomodulation, anti-lipid peroxidation^[6-9], and antitumor^[3]. RGE polysaccharides isolated from the dried root of RGE have also shown the properties of immunomodulation^[10-14] and antitumor^[15]. To few studies have investigated the effect of LBE and RGE extracts on HCC. The purpose of this study was to investigate the effect of crude hot water-extracted LBE and RGE on cell proliferation and apoptosis in HCC cells.

MATERIALS AND METHODS

Preparation of crude herbal extracts

LBE *L Radix* RGE (processed and dried RGE) were purchased from Chien Yuan Hang (Taipei, Taiwan). To maintain the quality and consistency of the ingredients, the crude extracts were prepared in a single batch with adequate quantity for this study. Dried LBE *L Radix* RGE (100 g) were incubated with 900 mL deionized water at 100°C for 2 h. The herbal juice was then centrifuged at 9000 r/min for 20 min to remove the precipitate. The remaining herbal juice was filtered with gauze. The filtered supernatant containing polysaccharides was precipitated with three volumes of 950 mL/L ethanol, concentrated (rotavapor R200 with glass assembly V; BÜCHI Labortechnik AG, Flawil, Switzerland) and lyophilized (freeze dry system Lyph-Lock 6; Labconco Corp., Kansas City, MO, USA)^[16].

Composition analysis of herbal extracts

The total carbohydrate content in hot water-extracted LBE and RGE was determined by phenol-sulfuric acid assay using glucose as a standard^[17]. The contents of crude protein, crude fat, moisture, and ash were measured using the Association of Official Analytical Chemists (AOAC) methods (981.10, 991.36, 925.10, 923.03)^[18].

Cell cultures and treatments

Rat (H-4-II-E, BCRC no. 60209) and human HCC (HA22T/VGH, BCRC no. 60168) cell lines were purchased from the Bioresources Collection and Research Center (BCRC) of the Food Industry Research and Development Institute (Hsinchu, Taiwan). Rat and human HCC cells (1×10^5 cells/mL) were grown in 850 mL/L minimum essential medium (MEM; GIBCO™, Invitrogen Corp., Carlsbad, CA, USA) with 150 mL/L fetal bovine serum (FBS; GIBCO™) or 900 mL/L Dulbecco's modified Eagle's medium (DMEM; GIBCO™) with 100 mL/L FBS, respectively, at 37°C in a humidified atmosphere of 950 mL/L air and 50 mL/L CO₂. Prior to addition of the treatment, the cells were grown to 80%-90% confluency and synchronized by incubating in serum-free basal medium (MEM or DMEM) for 24 h. The cells were then treated with various concentrations of crude LBE (0-5 g/L) or RGE (0-10 g/L) in the absence of serum for 0-24 h. The cells and medium were collected. Protein contents in the cells and medium were determined by the modified method of Lowry *et al.*^[19] using a Bio-Rad DC protein kit (Bio-Rad Laboratories, Hercules, CA, USA).

Cell proliferation assay

Cell proliferation was colorimetrically measured at 490 nm using a commercial proliferation assay kit (CellTiter 96® AQueous; Promega Corp., Madison, WI, USA). After treatment with various concentrations of crude LBE (0-5 g/L) or RGE (0-10 g/L) for 0-24 h, rat and human HCC cells ($n = 6$) in the 96-well plate were incubated with 20 µL MTS (3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) solution containing 1.90 g/L MTS and 300 µmol/L phenazine methosulfate in Dulbecco's PBS (pH 6.0) for 2 h at 37°C in a humidified 50 mL/L CO₂ atmosphere^[20]. The absorbance of soluble formazan produced by cellular reduction of MTS was determined at 490 nm using an ELISA reader (Multiskan RC; Labsystems, Helsinki, Finland).

Flow cytometric analysis of cellular DNA content

The percentage of cells undergoing apoptosis and distributing in different phases of cell cycle were determined by propidium iodide (PI)-staining method using flow cytometry. After treatment for 24 h, the conditioned medium of rat H-4-II-E cells ($n = 6$) was centrifuged at 800 r/min for 5 min at 4°C to remove the supernatant. The trypsinized cells and cell pellet of the conditioned medium were washed with PBS, fixed in 2 mL of 700 mL/L cold ethanol, and stored at 4°C overnight. After washed twice with PBS, the ethanol-fixed cells were incubated with 3 µL RNase (10 g/L) at 37°C for 30 min, and stained with 1 mL PI (40 mg/L) in the dark. The cell suspension was then filtered through a 35 µm mesh, and analyzed by a flow cytometer (FACSCalibur; Becton Dickinson Biosciences, San Jose, CA, USA) within 2 h. Cellular DNA content was calculated using CellQuest software (Becton Dickinson Biosciences).

Analysis of p53 protein

After incubation with various concentrations of LBE (0-5 g/L) or RGE (0-10 g/L) for 24 h, the conditioned medium of rat H-4-II-E cells was centrifuged at 800 r/min for 5 min at 4°C to remove the supernatant. Rat H-4-II-E cells and cell pellet in the conditioned medium were re-suspended in lysis buffer (2 mol/L Tris, 5 mol/L NaCl, 50 g/L NP-40, 100 g/L sodium dodecylsulfate (SDS), and 5 g/L phenylmethylsulfonyl fluoride), centrifuged at 300 r/min for 5 min at 4°C. The supernatant (40 µg total protein) pooled from 3 independent experiments ($n = 3$) was mixed with 4 × sample buffer (0.25 mol/L Tris-HCl, pH 6.8, 80 g/L SDS, 200 mL/L glycerol, 100 g/L β-mercaptoethanol, and 1 g bromophenol blue)^[21] denatured at 100°C for 3 min, and applied to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad Mini-PROTEAN 3 Cell; Bio-Rad Laboratories). Proteins were separated by 125 mL/L resolving gel (acrylamide: bisacrylamide = 37.5:1) with 40 mL/L stacking gel in the running buffer (25 mmol/L Tris, pH 8.3, 192 mmol/L glycine, and 1 g/L SDS) at 3 EV/cm per hour. Then the proteins were transferred onto nitrocellulose membrane (0.45 µm) using a semi-dry transfer unit (Hofer Semiphor

Table 1 Composition of hot water-extracted *Lycium barbarum* and *Rehmannia glutinosa*¹

Ingredient (mg/g)	<i>Lycium barbarum</i> extract	<i>Rehmannia glutinosa</i> extract
Carbohydrate	762.8	700.4
Protein	134.7	47.2
Fat	3.9	2.9
Moisture	221.6	92.4
Ash	0.5	0.4

¹The samples were at least triplicated.

TE 70, Amersham Biosciences Corp., San Francisco, CA, USA) in Towbin buffer (25 mmol/L Tris, 192 mmol/L glycine, 1 g/L SDS, and 100 mL/L methanol) at 6 mA/cm per hour^[22]. The membrane was washed with PBS, and incubated with a blocking buffer (50 g/L skim milk and 2 mL/L Tween-20 in PBS) for 2 h. Then the membrane was incubated with an anti-mouse monoclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) against p53 (Pab-246; 2 mg/L) or α -tubulin (TU-02; 1 mg/L) at room temperature for 2 h. Alpha-tubulin was used as an internal control. The membrane was washed three times with the wash buffer (2 mL/L Tween-20 in PBS), and incubated with 4 mg/L goat anti-mouse IgG-horseradish peroxidase conjugate (Santa Cruz Biotechnology, Inc.) for 2 h. The blot was washed three times again with the wash buffer, incubated with luminol reagent (PerkinElmer Life and Analytical Sciences, Inc., Boston, MA, USA) for 2 min, and exposed to an X-ray film (Eastman Kodak Co., Rochester, NY, USA) for 3-5 min. The bands were quantitated by an image analysis system (0.2-megapixel charge-coupled device camera, gel analysis system; EverGene Biotechnology, Taipei, Taiwan) and Phoretix 1D Lite software (version 4.0; Phoretix International Ltd., Newcastle upon Tyne, UK).

Statistical analysis

Data are expressed as mean \pm SD. Data were analyzed by one- and two-way analysis of variance (ANOVA) to determine the treatment effect using the Statistical Analysis System (SAS version 8.2; SAS Institute Inc., Cary, NC, USA). Fisher's least significant difference test was used to make *post hoc* comparisons if the treatment effect was demonstrated. $P < 0.05$ was considered statistically significant.

RESULTS

Composition analysis of herbal extracts

The extraction rate for crude LBE and RGE was 704 mg/g and 724 mg/g, respectively. Carbohydrate content, the major component, was 762.8 mg/g and 700.4 mg/g in crude LBE and RGE, respectively (Table 1). Moisture and protein contents in crude LBE and RGE were 221.6 mg/g and 134.7 mg/g as well as 92.4 mg/g and 47.2 mg/g, respectively. Fat and ash contents in these crude extracts were less than 5 mg/g.

Cell proliferation assay

Crude LBE and RGE at a dose of 1 g/L did not inhibit

Table 2 Effect of hot water-extracted *Lycium barbarum* (LBE) and *Rehmannia glutinosa* (RGE) on cell proliferation in rat hepatocellular carcinoma (H-4-II-E) cells ($n = 6$, mean \pm SD)

	Cell proliferation (% of control)			
	6 h	12 h	18 h	24 h
Control	100.0 \pm 0.7 ^{h,j}	100.0 \pm 8.6 ^{e,h,j}	100.0 \pm 5.9 ^{f,h,j}	100.0 \pm 5.9 ^{f,h,j}
1 g/L LBE	100.3 \pm 9.8 ^{f,h}	100.7 \pm 8.2 ^{e,h}	100.1 \pm 5.7 ^{f,h}	97.8 \pm 6.3 ^{f,h}
2 g/L LBE	84.4 \pm 6.6 ^{b,d,h}	89.3 \pm 7.5 ^{a,c,h}	83.8 \pm 7.5 ^{b,d,h}	86.3 \pm 2.9 ^{b,d,h}
5 g/L LBE	37.6 \pm 6.9 ^{b,d,f}	23.8 \pm 4.6 ^{b,d,f}	18.0 \pm 5.3 ^{b,d,f}	15.1 \pm 3.1 ^{b,d,f}
1 g/L RGE	99.5 \pm 7.1 ^{f,h,j}	97.0 \pm 13.4 ^{b,j}	111.4 \pm 11.7 ^{a,f,h,j}	96.1 \pm 5.3 ^{f,h,j}
2 g/L RGE	80.9 \pm 18.4 ^{b,d,h,j}	88.3 \pm 9.4 ^{a,h,j}	84.6 \pm 14.4 ^{b,d,h,j}	84.4 \pm 6.4 ^{b,d,h,j}
5 g/L RGE	58.3 \pm 11.4 ^{b,d,f,j}	74.6 \pm 7.0 ^{b,d,f,j}	57.1 \pm 10.1 ^{b,d,f,j}	71.2 \pm 6.7 ^{b,d,f,j}
10 g/L RGE	29.2 \pm 6.9 ^{b,d,f,h}	29.5 \pm 6.3 ^{b,d,f,h}	25.0 \pm 5.7 ^{b,d,f,h}	24.2 \pm 7.1 ^{b,d,f,h}

^a $P < 0.05$, ^b $P < 0.01$ vs control, ^c $P < 0.05$, ^d $P < 0.01$ vs 1 g/L corresponding treatment, ^e $P < 0.05$, ^f $P < 0.01$ vs 2 g/L corresponding treatment; ^g $P < 0.05$, ^h $P < 0.01$ vs 5 g/L corresponding treatment, ⁱ $P < 0.01$ vs 10 g/L corresponding treatment within the same column.

Table 3 Effect of hot water-extracted LBE and RGE on cell proliferation in human hepatocellular carcinoma (HA22T/VGH) cells after 24 h incubation ($n = 6$, mean \pm SD)

	Cell proliferation (% of control)
Control	100.0 \pm 4.2 ^{d,f,h}
2 g/L LBE	86.0 \pm 5.9 ^{b,c,h}
5 g/L LBE	77.0 \pm 5.1 ^{a,b,h}
10 g/L LBE	56.8 \pm 3.9 ^{b,d,f}
2 g/L RGE	86.3 \pm 5.9 ^{b,f,h}
5 g/L RGE	74.3 \pm 7.5 ^{b,d}
10 g/L RGE	70.3 \pm 7.7 ^{b,d}

^b $P < 0.01$ vs control; ^a $P < 0.05$, ^d $P < 0.01$ vs 2 g/L corresponding treatment; ^c $P < 0.05$, ^f $P < 0.01$ vs 5 g/L corresponding treatment; ^h $P < 0.01$ vs 10 g/L corresponding treatment.

cell proliferation up to 24 h incubation in rat H-4-II-E cells (Table 2). However, crude RGE at a dose of 1 g/L slightly increased cell proliferation of H-4-II-E cells at 18 h incubation compared with the control group (111.4% \pm 11.7% vs 100.0% \pm 5.9% of control, $P = 0.03 < 0.05$). Crude LBE (2-5 g/L) and RGE (2-10 g/L) dose-dependently inhibited cell proliferation by 11% ($P < 0.05$) to 85% ($P < 0.01$) compared with the control group after 6-24 h incubation. The inhibitory effect of crude LBE and RGE at lower doses (1-2 g/L) on cell proliferation was not significantly different. A higher dose (5 g/L) of crude LBE suppressed cell proliferation more efficiently ($P < 0.01$) than that of crude RGE. From the curves of four time points, the mean values of IC50 for crude LBE and RGE were 3.8 g/L and 6.9 g/L, respectively. Similar results in human HA22T/VGH cells, crude LBE (2-10 g/L) and RGE (2-5 g/L) dose-dependently inhibited cell proliferation by 14%-43% ($P < 0.01$) compared with the control group after 24 h incubation (Table 3). Cell proliferation was inhibited equivalently by crude LBE and RGE at lower doses (2-5 g/L). However, crude LBE at a higher dose (10 g/L) showed better suppression (56.8% \pm 3.1% vs 70.3% \pm 3.1% of control, $P = 0.0003 < 0.01$) on cell proliferation compared with the same dosage of crude RGE. The IC50 value for both crude LBE and RGE was above 10 g/L.

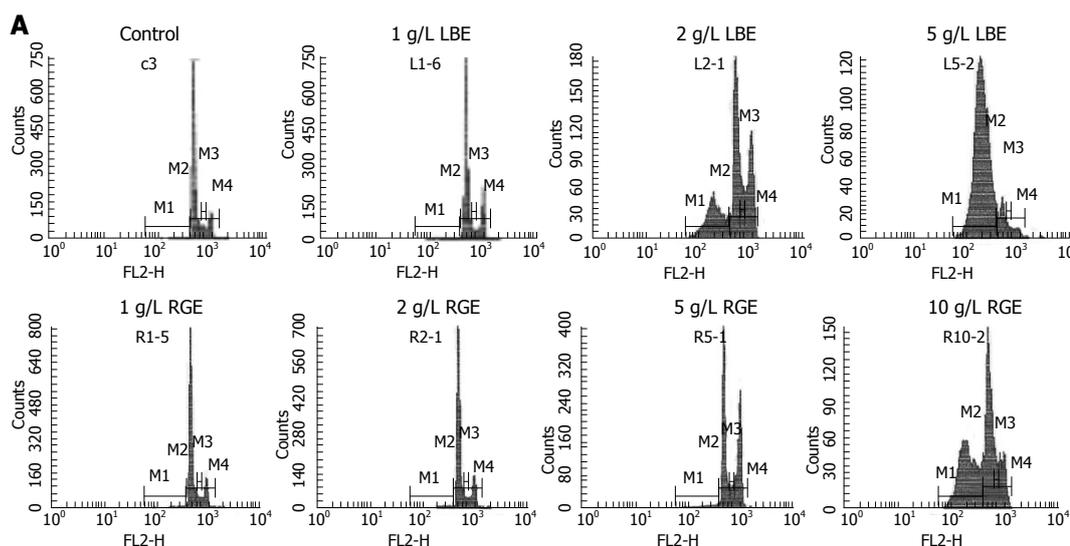
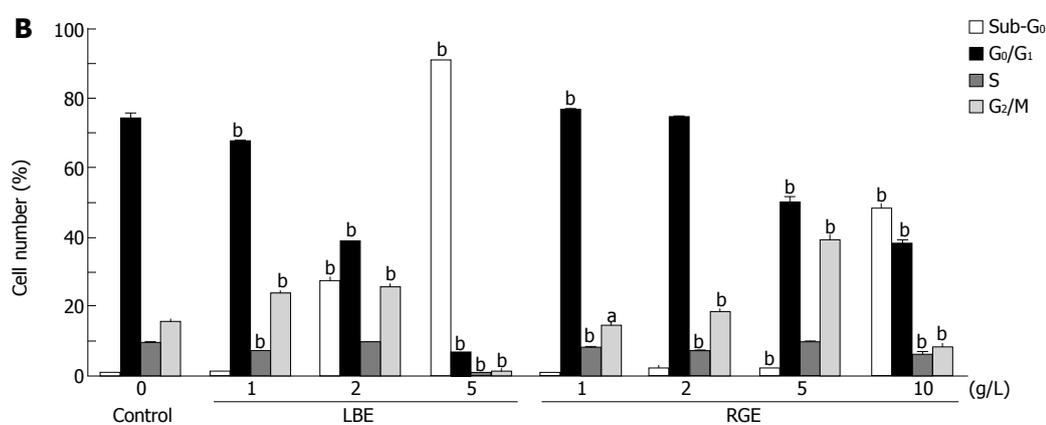


Figure 1 Representative DNA histograms (A) and percentage of cells in different cell cycle phases (B) after incubated with various concentrations of hot water-extracted LBE and RGE for 24 h in rat H-4-II-E cells determined by flow cytometry. Values are mean \pm SD ($n = 6$). (□), sub-G₀ phase (M1 peak); (■), G₀/G₁ phase (M2 peak); (▒), S phase (M3 peak); (▫), G₂/M phase (M4 peak), ^a $P < 0.05$, ^b $P < 0.01$ vs control within the same cell cycle phase.



Flow cytometric analysis of cellular DNA content

Cellular DNA content in cell cycle distribution was determined, and apoptosis was quantitated by the percentage of cells with sub-G₀ DNA content by flow cytometry (Figure 1A). Crude LBE and RGE at the doses above 2 g/L and 5 g/L, respectively, significantly increased the percentage of cells in sub-G₀ phase (27.5% \pm 0.5% and 91.2% \pm 0.3% for 2 g/L and 5 g/L LBE, and 2.4% \pm 0.2% and 48.1% \pm 2.0% for 5 g/L and 10 g/L RGE, $P < 0.01$) compared with the control group (1.0% \pm 0.2%) (Figure 1B). The percentage of cells in G₀/G₁ phase was dose-dependently decreased by crude LBE and RGE. Crude LBE at lower doses (1-2 g/L) significantly increased the percentage of cells in G₂/M phase (24.1% \pm 0.4% and 25.8% \pm 0.3%, $P < 0.01$) compared with the control group (15.8% \pm 0.4%), but decreased the percentage of cells to 1.5% \pm 0.1% ($P < 0.01$) at a higher dose (5 g/L). Likewise, crude RGE at lower doses (2 g/L and 5 g/L) significantly increased the percentage of cells in G₂/M phase (18.3% \pm 0.4% and 39.3% \pm 2.1%, $P < 0.01$), but decreased the percentage of cells to 8.3% \pm 0.7% ($P < 0.01$) at a higher dose (10 g/L).

Analysis of p53 protein

After 24 h incubation with crude LBE and RGE, the expression of p53 protein in rat H-4-II-E cells was analyzed by SDS-PAGE and Western blotting (Figure 2A). After calibrated by an internal control (α -tubulin), the expression of p53 protein increased with the dosage of crude

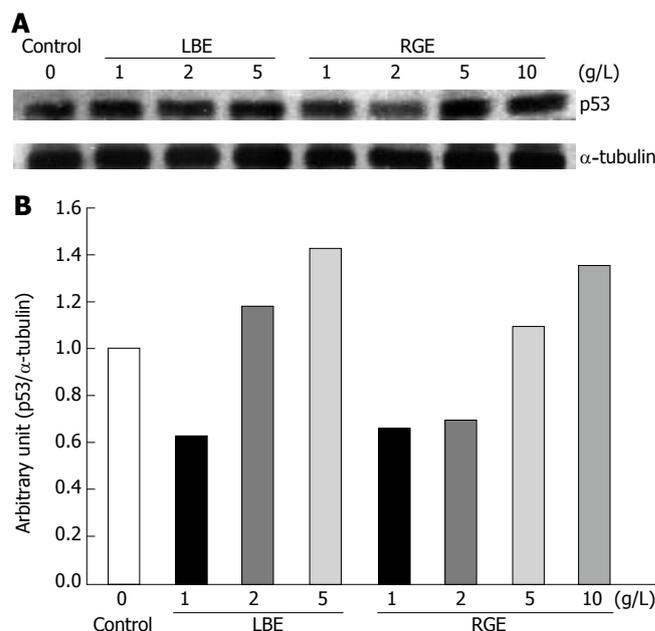


Figure 2 Expression of p53 protein with the molecular weight of 53 ku visualized by Western blotting (A) and quantitated by an image analysis system (B) after incubation of rat H-4-II-E cells with various concentrations of hot water-extracted LBE and RGE for 24 h. Samples were pooled from 3 independent experiments ($n = 3$). Alpha-tubulin (55 ku) was used as an internal control.

LBE and RGE (Figure 2B). Crude LBE at higher doses (2-5 g/L) increased p53 expression to 119% and 143% of the

control group, respectively. Similarly, crude RGE at higher doses (5-10 g/L) increased p53 expression to 110% and 136% of the control group, respectively. However, lower doses of crude LBE (1 g/L) and RGE (1-2 g/L) suppressed p53 expression to 63%-70% of the control group.

DISCUSSION

Polysaccharides, a water-soluble bioactive component from LBE *L Radix* RGE, showed antitumorigenic activity^[3,15]. The methods for the isolation of polysaccharides from these herbs are various in different studies. Different extraction methods and fractions could affect not only the ingredients but also the physiological activity of the extract. Our crude herbal extracts were prepared by hot water (100°C) extraction followed by ethanol (950 mL/L) precipitation. Gan *et al*^[3] extracted LBE with distilled water at 80°C after removal of pigment by acetone/petroleum (1:1) and oligosaccharides by 800 mL/L ethanol. The crude polysaccharide-protein complex was precipitated by ethanol, and the acidic fraction was further purified by diethylaminoethyl-cellulose anion exchange and Sephadex G200 column chromatography. Finally, a purified fraction (LBP_{3p}) contains 63.6% neutral sugars, 24.8% acidic sugars, and 7.6% proteins. The purified fraction showed antiproliferating and immunomodulating activity in S180-bearing mice. Compared with the purified LBP_{3p} fraction, our crude LBE contains lower sugars (88.4% *vs* 76.3%) and higher proteins (7.6% *vs* 13.5%). Zhang *et al*^[4] prepared purified fraction of RGE oligosaccharides by extraction with hot water and isolation with cation/anion exchange and charcoal column chromatography. The eluted fraction containing monosaccharides, disaccharides, trisaccharides, and other oligosaccharides but not polysaccharides exert a significant hypoglycemic effect in normal and alloxan-induced diabetic rats. Our crude extract did not contain certain water-insoluble active components in LBE and RGE. Scopoletin (7-hydroxy-6-methoxycoumarin), slightly soluble in water, could be excluded as extracted by hot water, and has been reported as an active component of the fruit of LBE for inhibiting cell proliferation of human prostate cancer PC3 cells^[23]. Furan derivatives, isolated from chloroform extract of the dried roots of RGE, have the immunomodulating and anti-coagulating activity^[24].

Our study found that both crude LBE and RGE suppressed cell proliferation in rat and human HCC cells in a dose-dependent manner. Consistent with our findings, Zhang *et al*^[25] showed that polysaccharide containing LBE extract inhibits cell proliferation of human hepatoma QGY7703 cells. Additionally, LBE polysaccharides (20-1000 mg/L) dose-dependently suppress cell growth of human leukemia HL-60 cells^[26]. The previous *in vivo* studies also showed that LBE exhibits the antitumorigenic activity in tumor-bearing mice^[3,27]. A polysaccharide-protein complex from LBE (LBP_{3p}) at 10 µg/g significantly reduces tumor weight and enhances immune functions in S180-bearing mice^[3]. RGE polysaccharide b with an average molecular weight of 160 ku has antitumorigenic and immunomodulating activity in S180-bearing mice^[13]. A clinical trial found 40.9% response rate in the advanced cancer patients

with lymphokine-activated killer (LAK) cells and interleukin-2 (IL-2) treatment combined with LBE polysaccharides compared with 16.1% response rate ($P < 0.05$) in those treated with LAK/IL-2 alone. These data indicate that LBE polysaccharides can be used as an adjuvant for the treatment of cancer^[28]. Chinese medicinal herbs containing RGE have been demonstrated to alleviate the adverse effects of high-dose methotrexate plus vincristine in post-operative osteogenic sarcoma patients with chemotherapy, suggesting that Chinese medicinal herbs containing RGE are less toxic compared with a traditional chemotherapy^[29]. The extract of LBE at the dose of 25 g/L and 5 g/L inhibited cell proliferation by 14% and 85%, and stimulated p53 protein expression by 19% and 43% in rat H-4-II-E cells after 24 h incubation. The extract of RGE at the dose of 5 g/L and 10 g/L inhibited cell proliferation by 23% and 76%, and stimulated p53 protein expression by only 10% and 36% in rat H-4-II-E cells after 24 h incubation. The inhibition of cell proliferation by LBE at the dose of 2 g/L was almost parallel to the stimulation of p53. However, the relationship between cell proliferation inhibition and p53 expression was not obvious in rat H-4-II-E cells treated with higher doses of LBE and RGE.

The percentage of cells in sub-G₀ phase significantly increased in H-4-II-E cells after 24 h treatment with higher doses of crude LBE (2-5 g/L) and RGE (5-10 g/L). A dramatic increased cell percentage in sub-G₀ phase by crude LBE and RGE at higher doses was probably due to late apoptotic/necrotic cells in sub-G₀ phase if undergoing autolysis, which could overestimate the apoptotic cells. The proliferation-inhibiting and apoptosis-inducing activity of crude LBE at higher doses (≥ 5 g/L) in HCC cells was more effective than that of crude RGE according to the mean values of IC50 and the proportion of apoptotic cells. As initiating a significant increase in the percentage of cells in sub-G₀ phase by crude LBE (2 g/L) and RGE (5 g/L), the percentage of cells in G₂/M phase significantly increased. Additionally, the expression of p53 protein was correspondently stimulated as well after 24 h incubation with 2 g/L crude LBE and 5 g/L crude RGE. However, lower doses of crude LBE and RGE decreased the expression of p53 protein compared with the control group, which could be due to the overexposure to α -tubulin. The expression of p53 protein tended to be dose-dependently increased by crude LBE and RGE. The results suggest that higher doses of crude LBE and RGE arrest cells in G₂/M phase and p53 may be involved in mediating apoptosis. The mechanism of promoting G₂/M arrest by LBE and RGE has not been understood. It is proposed that LBE and RGE may inhibit nuclear factor- κ B to alter the expression of regulatory cell cycle proteins such as cyclin B and/or p21WAF1/Cip1. A previous study reported that LBE polysaccharides arrest cells in S phase and induce apoptosis with increased intracellular calcium in human hepatoma QGY7703 cells^[25]. Additionally, LBE (20-1000 mg/L) results in DNA fragmentation and positive TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) signals in human leukemia HL-60 cells, indicating that LBE induces apoptosis^[26]. Although the effect of LBE and RGE on apoptosis in normal hepatocytes has not been studied yet, the previous studies^[30,31] found

that LBE protects normal cells from apoptosis rather than stimulating apoptosis in tumor cells. LBE dose-dependently inhibits apoptosis induced by hydrocortisone in rat spleen *in vitro*^[30]. LBE extract shows cytoprotective effect against β -amyloid peptide-induced apoptosis in primary rat cortical neurons and dithiothreitol-induced caspase-3 activation in normal neural endoplasmic reticulum^[31]. The effect of LBE on apoptosis could be various due to different extraction methods, extraction fractions, dosages, tissues, and cell types (normal *vs* malignant). The low-molecular-weight RGE polysaccharides at the doses of 20 $\mu\text{g/g}$ and 40 $\mu\text{g/g}$ markedly increase the level of p53 mRNA to 3.3- and 3.2-fold, respectively, in Lewis lung cancer tissue of C57BL/6 mice^[15]. Activation of p53 tumor suppressor protein can lead to cell cycle arrest and apoptosis in response to DNA damage^[32]. Cell death induced through p53-mediating pathway is subsequently initiated by the activation of caspases followed by the characteristic apoptotic phenotype.

The mechanisms underlying regulation of apoptosis by LBE and RGE have not been clearly understood. Besides the induction of p53-mediated apoptosis, immunomodulation may contribute to antitumorigenesis. A polysaccharide-protein complex from LBE (LBP_{3p}) dose-dependently increases the expression of IL-2 and tumor necrosis factor (TNF)- α at the levels of mRNA and protein in human peripheral blood mononuclear cells^[33]. An *in vivo* study showed that LBP_{3p} administered orally at 10 $\mu\text{g/g}$ for 10 d inhibits cell growth of transplantable sarcoma S180 cells as well as increases macrophage phagocytosis, cell proliferation of spleen lymphocytes, the activity of cytotoxic T lymphocytes (CTL), and the expression of IL-2 mRNA in S180-bearing mice^[3]. RGE polysaccharide b at the intraperitoneal injection dose of 10 $\mu\text{g/g}$ or 20 $\mu\text{g/g}$ attenuates the decrease in CTL activity caused by excessive tumor growth through increasing the production of CD8⁺ (Lyt-2⁺) CTL and lowering the ratio of CD4⁺ to CD8⁺ (L3T4⁺) T lymphocyte subset in S180-bearing mice^[13]. An aqueous extract of RGE-steamed root dose-dependently suppresses the secretion of TNF- α by mouse astrocytes stimulated with substance P and lipopolysaccharide through the inhibition of IL-1 secretion, suggesting that RGE has the anti-inflammatory activity^[14]. Although crude LBE and RGE show antitumorigenic activity *in vitro*, it still remains to determine the antitumorigenic components of crude LBE and RGE, and molecular mechanisms for regulating the proliferation of HCC cells.

In conclusion, hot water-extracted LBE (2-5 g/L) and RGE (5-10 g/L) inhibit proliferation and stimulate apoptosis probably involved in p53 mediation in HCC cells. It is not known that whether hot water-extracted LBE and RGE have antiproliferative and apoptosis-stimulating effects on HCC *in vivo* and further studies are still required to verify their *in vivo* effects on both normal and malignant hepatocytes.

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Risk of colon cancer in hereditary non-polyposis colorectal cancer patients as predicted by fuzzy modeling: Influence of smoking

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may enable formulation of clinical risk scores, thereby allowing individualization of CRC prevention strategies.

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Abstract

AIM: To investigate whether a fuzzy logic model could predict colorectal cancer (CRC) risk engendered by smoking in hereditary non-polyposis colorectal cancer (HNPCC) patients.

METHODS: Three hundred and forty HNPCC mismatch repair (MMR) mutation carriers from the Creighton University Hereditary Cancer Institute Registry were selected for modeling. Age-dependent curves were generated to elucidate the joint effects between gene mutation (hMLH1 or hMSH2), gender, and smoking status on the probability of developing CRC.

RESULTS: Smoking significantly increased CRC risk in male hMSH2 mutation carriers ($P < 0.05$). hMLH1 mutations augmented CRC risk relative to hMSH2 mutation carriers for males ($P < 0.05$). Males had a significantly higher risk of CRC than females for hMLH1 non smokers ($P < 0.05$), hMLH1 smokers ($P < 0.1$) and hMSH2 smokers ($P < 0.1$). Smoking promoted CRC in a dose-dependent manner in hMSH2 in males ($P < 0.05$). Females with hMSH2 mutations and both sexes with the hMLH1 groups only demonstrated a smoking effect after an extensive smoking history ($P < 0.05$).

CONCLUSION: CRC promotion by smoking in HNPCC patients is dependent on gene mutation, gender and age. These data demonstrate that fuzzy modeling

INTRODUCTION

Accurate risk-stratification is essential for combating the 50000 yearly deaths from colorectal cancer (CRC) in the United States^[1]. The best-established risk factor is a familial predisposition to CRC, which is implicated in one-quarter of all CRC cases^[2]. While determining a family history can be readily accomplished, risk quantification which is critical for tailoring screening strategies, remains remarkably imprecise. For instance, even in documented carriers of CRC predisposing genes, clinical expression can be quite varied due to modulation by numerous confounding endogenous and exogenous variables^[3,4].

Hereditary non-polyposis colorectal cancer (HNPCC) represents a case in point. This autosomal dominant condition is the most common cancer predisposing syndrome engendering a > 70% lifetime risk of developing CRC^[5]. Furthermore, we have recently demonstrated that cigarette smoking of a male carrying hMLH1 mutations (versus hMSH2) increases the hazard of CRC by 1.4-, 1.6- and 2.0-fold respectively^[6]. However, optimal management strategies (colonoscopic surveillance versus prophylactic colectomy) are unclear secondary to characteristic phenotypic heterogeneity, i.e. marked variations in age of onset of cancers^[7]. Thus, HNPCC represents an excellent paradigm to study the gene-environment joint effect hypothesis.

Incorporating these important findings into clinical

practice is hindered by the inability to accurately quantitate the risk modulation engendered by the joint effects of genetics and environmental factors. Moreover, the inadequacy of conventional statistical approaches to model the complex nature of many of the CRC risk factors further limits application of these data to patient management. While landmark studies have explored the age of CRC diagnosis in HNPCC^[8], these estimates along with others in the literature have not yet factored in the genetic/environmental influences that determine the phenotypic heterogeneity. The focus of our study was on this phenotypic heterogeneity which is of major importance to clinicians who care for these challenging patients. We believe that by knowing the genetic and environmental risk factors, we can individualize more accurately the risk analysis, which, to our knowledge, has not been previously reported.

One approach from the engineering literature that has recently received attention for cancer risk assessment is fuzzy logic. This powerful modeling technique has been successfully used for pattern recognition and image processing and its unique ability to transcend the typical black or white approaches in standard modeling and to capture the “shades of gray” has great promise for clinical medicine^[9]. While typical statistical approaches function well when the data are normally distributed and values are near the mean, this approach is often inadequate at the threshold. For instance, a very high prostate specific antigen (PSA) has excellent predictive ability for prostate cancer, but the optimal clinical management of a patient with a mildly elevated value is unclear. Fuzzy logic overcomes these limitations of conventional statistics by allowing partial membership function. In our PSA example, instead of categorizing values as either normal or abnormal, a fuzzy approach would allow one to place a value as one quarter in the normal group and three-quarters in the abnormal group. Thus, through the creation of fuzzy sets, elements can have degrees of membership on a continuum (e.g. a value can be “normal, slightly elevated, moderately elevated or highly elevated”).

Another unique attribute of fuzzy modeling is that, unlike traditional models, it does not require prior knowledge of the system being modeled. It is a “model-free” form in which natural rules are developed from the data rather than imposing rules on the modeling system. The result of this “model-free” system is still a conversion from inputs to outputs, similar to traditional algorithms^[10]. Another strength of fuzzy modeling lies in its ability to model data points that may be outside of traditional inclusion boundaries and thus allowing accurate modeling with less data.

Past reports have demonstrated that fuzzy modeling can improve performance characteristics of tumor markers over conventional applications^[11-13]. Our previous work with conventional statistical approaches (COX proportional hazard modeling) indicated that tobacco use, gender and mutated gene play an important role in phenotypic presentation of CRC progression in HNPCC patients as a group, but lack the ability to predict an individual risk of CRC (e.g. not sensitive to dose effects or interactions of factors). We, therefore, explored the

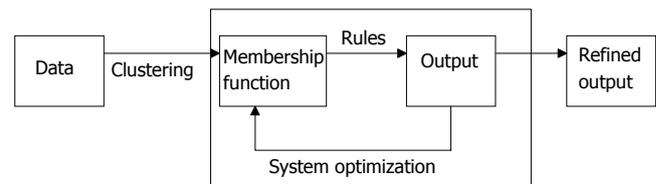


Figure 1 Overview of fuzzy methodology.

ability of fuzzy modeling to predict CRC risk in germline mutation carriers in these individual HNPCC patients by factoring the gene type, gender and tobacco use status in the present study.

MATERIALS AND METHODS

Database

The Hereditary Cancer Center at Creighton University is one of the oldest and largest registries for diverse hereditary cancer syndrome, containing information on over 200 000 individuals of whom approximately 600 are verified MMR mutation carriers (Lynch Database). The database contains patient and family information, surveillance and treatment information as well as gene mutations and lifestyle data. The inclusion criteria were HNPCC as documented by either a MMR germline mutation positivity or clinical HNPCC from a patient who had a family member with a documented MMR mutation. For example, if the patient has HNPCC and his mother has a documented hMSH2 mutation, we would consider the patient to have an hMSH2 mutation. Tobacco data were obtained by self-report and family report or by abstraction from medical records. A patient was classified as a tobacco user if he/she reported ever regularly using (or was reported to have ever regularly used) cigarettes, cigars, a pipe, tobacco chew, or snuff. Five hundred and ninety-six mutation carriers were identified from 62 HNPCC families. For this analysis we only focused on cigarette smokers. Of these, 340 (60.4%) had information on tobacco use and were included in our study (158 nonsmokers and 182 smokers). In a further analysis, 271 patients (113 of 182 smokers and 158 nonsmokers) with a more detailed smoking history including calculated pack-years were selected.

Modeling

The entire modeling procedure was performed using the MatLab Fuzzy Toolbox (Matlab, Version 6.1-Release 13, Natick, MA). Figure 1 provides an overview of the basic fuzzy modeling algorithm. The data (either categorical or continuous) were inputted into the program and a clustering algorithm led to the development of membership functions. The fuzzy clustering method used produces descriptions of each of the input vectors as belonging to one (or more) fuzzy sets with a specific membership in each of the sets. The inclusion of a continuous variable (pack-years and age) produces non-categorical (aka, fuzzy) memberships. Rules are then developed from these membership functions which successfully produce a mapping from the input space to the output space as previously described^[10]. Furthermore,

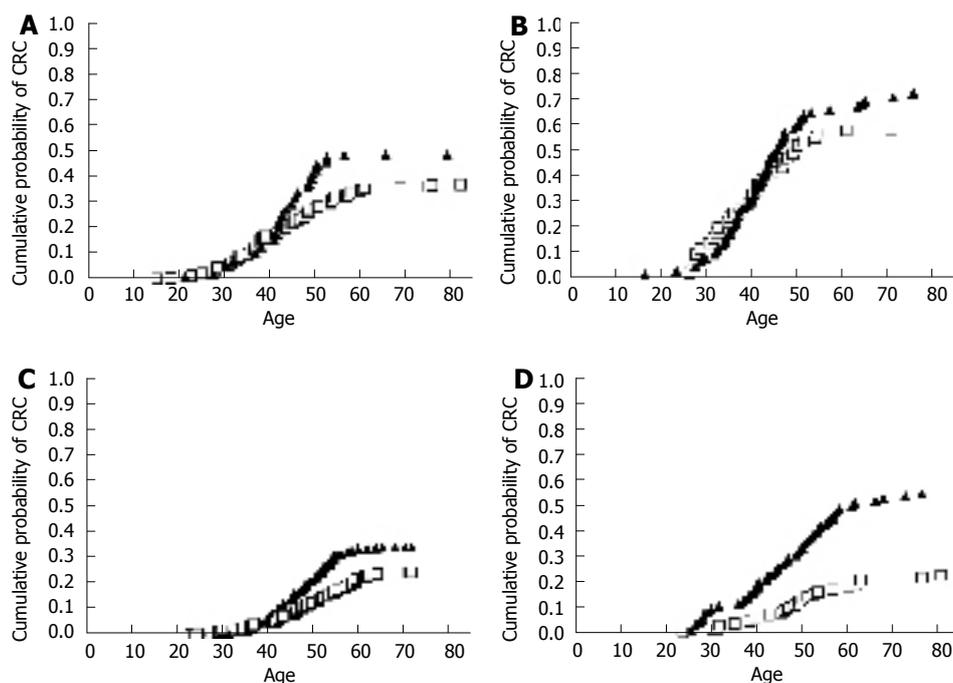


Figure 2 Scattergram demonstrating the probability of developing CRC (cumulative lifetime risk) for each subject based on smoking status, \blacktriangle = Smokers, \square = Non-Smokers. Data are divided by gene mutation and sex with (A) hMLH1: Female, (B) hMLH1: Male, (C) hMSH2: Female and (D) hMSH2: Male ($P < 0.05$).

these rules represent fuzzy relationships between the variables, even if the variables themselves are categorical. The refinement of these rules is accomplished using the Adaptive Neural Fuzzy Inference System (ANFIS; 16) which acts as a feedback loop to further refine the rules until they are optimized to give the best fit to the data. Overfitting of the model to the data is not exclusively addressed. However, the clustering methods (subtractive clustering) employed in the modeling scheme tend to partition the data space in such a manner as to maximize the cluster density while simultaneously maximizing the separation of the clusters which would limit overfitting.

This modeling technique was applied to the patients selected from the Lynch database with gene mutation, sex, smoking status and age as the input and risk of developing colorectal cancer as the output. In a second study, the effect of pack years was added as an additional input. In this case, dividing a group of smokers with a given mutation, sex and age further by smoke years made the numbers in each group quite small. We therefore used the data as the training set and a theoretical set of conditions as the input to generate the model output. The models produced a cumulative risk of CRC that ranged between 0 and 1. Age and pack years were fuzzified in the program.

Statistical analysis

Results are presented either as a scattergram of the actual model output for each patient in the database (Figures 2-4) or as an output of the model given a set of theoretical conditions (Figure 5). The statistical procedures used followed the methods described by Steel and Torrie^[14]. The data for each cohort were paired and compared using a Kolmogorov-Smirnov (KS) 2-sample test. The KS test was considered to be conservative and useful when hypothesis about discrete distributions was tested. The test is motivated by the need to compare 2 independent samples and the null hypothesis is that each sample originates from

identical distributions (i.e., the data are from the same population). Critical values for the KS test are inversely proportional to the square root of the total number of observations. The nature of our data dictated that the critical values were computed using unequal populations ($n_1 \neq n_2$). Furthermore, the nature of the data and results only required a comparison of this type. Other analyses, such as a test of trend or analysis of variance, were not deemed beneficial.

RESULTS

Smoking status

The influence of genetic mutation combined with sex, smoking status and age is demonstrated in Figure 2. There was a clear impact of cigarette smoking on the age-adjusted risk of developing CRC for all conditions tested (gene mutated and gender).

When a male patient with the hMSH2 gene mutation carrier smokes, he markedly increased his risk of developing CRC by up to 2.4-fold at the age of 78 (Figure 2D, $P < 0.05$). In the case of a mutation in the hMLH1 gene, smoking increased the risk of CRC at the maximal age tested by approximately 1.3-fold for males when compared to non-smokers (Figure 2B). Females with the hMLH1 mutation showed a 1.3-fold increased risk of developing CRC and female smokers with the hMSH2 mutation had a 1.4-fold greater risk of developing CRC when compared to their non-smoking age-matched controls (Figures 2A and 2C).

Gene

The model output was then reexamined as a function of the genetic mutation (Figure 3). For males, patients with a hMLH1 mutation had either a 2.5- or a 1.3-fold greater risk of developing CRC than those with a hMSH2 for non-smokers and smokers respectively (Figures 3B and 3D, $P <$

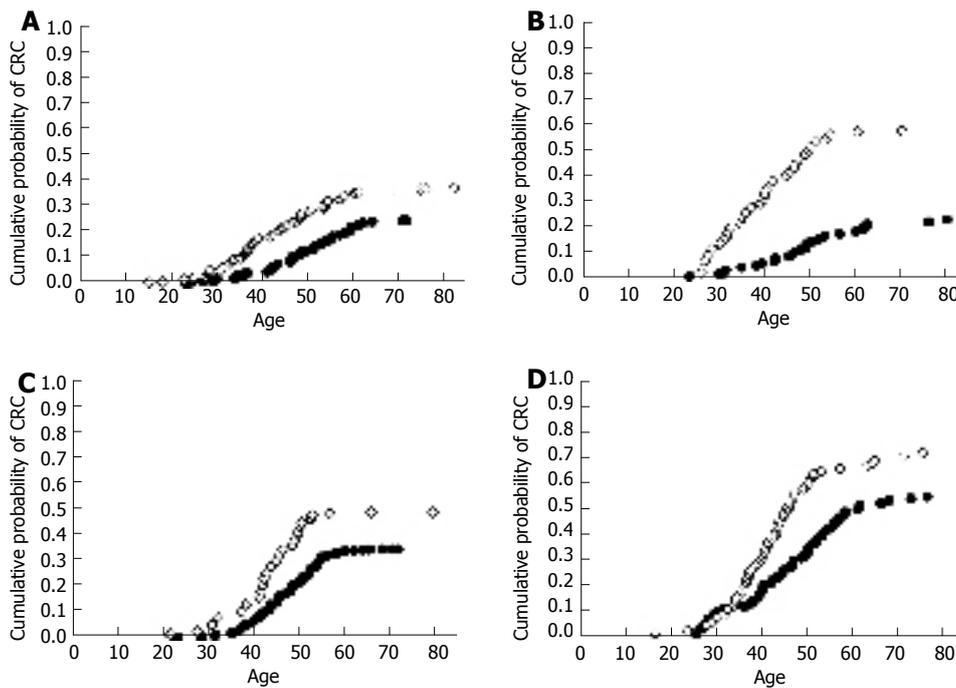


Figure 3 Scattergram demonstrating the probability of developing CRC (cumulative lifetime risk) for each subject based on genetic mutation, \diamond = hMLH1, \bullet = hMSH2. Data are divided by sex and smoking status with (A) Female: non-smokers ($P < 0.001$), (B) Female: smokers ($P < 0.001$), (C) Male: non-smokers ($P < 0.001$) and (D) Male: smokers ($P < 0.05$). The P values show the probability failing to reject the null hypothesis that the data originate from identical populations, indicating that the curves are different.

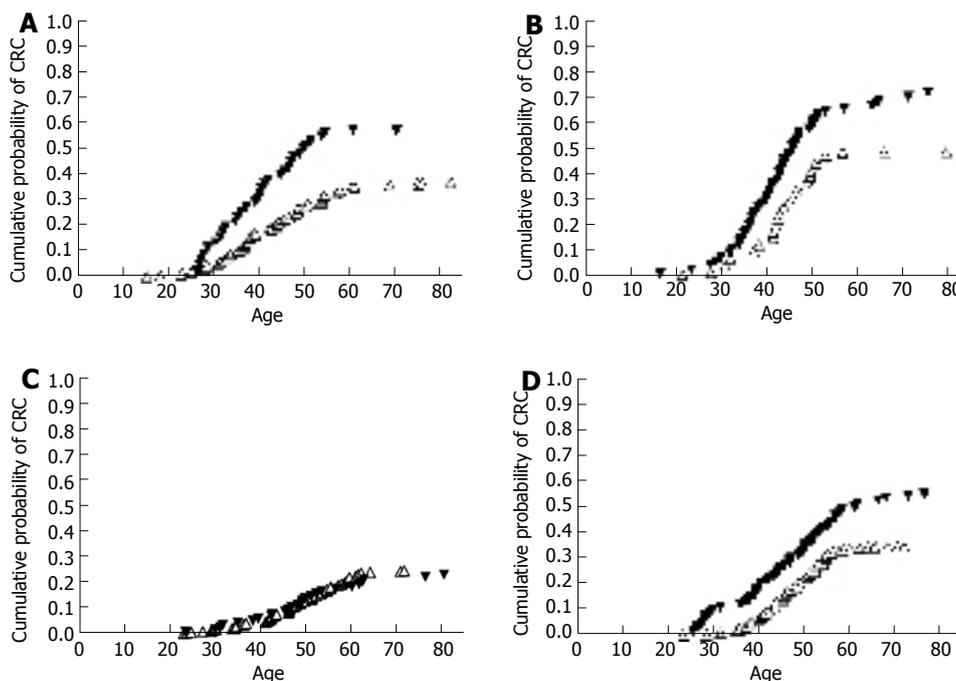


Figure 4 Scattergram demonstrating the probability of developing CRC (cumulative lifetime risk) for each subject based on gender, \triangle = female, ∇ = male. Data are divided by gene mutation and smoking status with (A) hMLH1: non-smokers, (B) hMLH1: smokers ($P < 0.1$), (C) hMSH2: non-smokers ($P > 0.05$) and (D) hMSH2: smokers ($P < 0.1$). The P values show the probability of failing to reject the null hypothesis that the data originate from identical populations, indicating that the curves are different.

0.05). The difference was greater for the non-smokers than the smokers because subjects with hMSH2 who smoke increased their rate of CRC greater than the non-smokers. Female non-smokers with the hMLH1 mutation showed a 1.5-fold increase in CRC risk as compared to the hMSH2 subjects, whereas it was 1.4-fold higher for smokers.

Gender

Figure 4 demonstrates that males had a greater risk of developing CRC than females when compared to an equivalent age, gene mutation and smoking status. Males had a significantly higher risk of CRC than females (1.5-fold) for non-smoking subjects with the hMLH1

mutations (Figure 4A, $P < 0.05$) and smokers (1.6-fold) (Figure 4B, $P < 0.1$). Smoking males with the hMSH2 mutation also had a greater risk of CRC than hMSH2 mutation harboring females by 1.6-fold (Figure 4D, $P < 0.01$), but the gender effect dissipated in nonsmokers (Figure 4C, $P > 0.05$).

Smoking dose response

A subset of the data which had an estimate of pack years was then modeled to determine if there was an association between lifetime quantities of cigarettes smoked and risk of CRC. The mean consumption was 24-pack years for patients who smoked cigarettes. Figure 5 demonstrates the

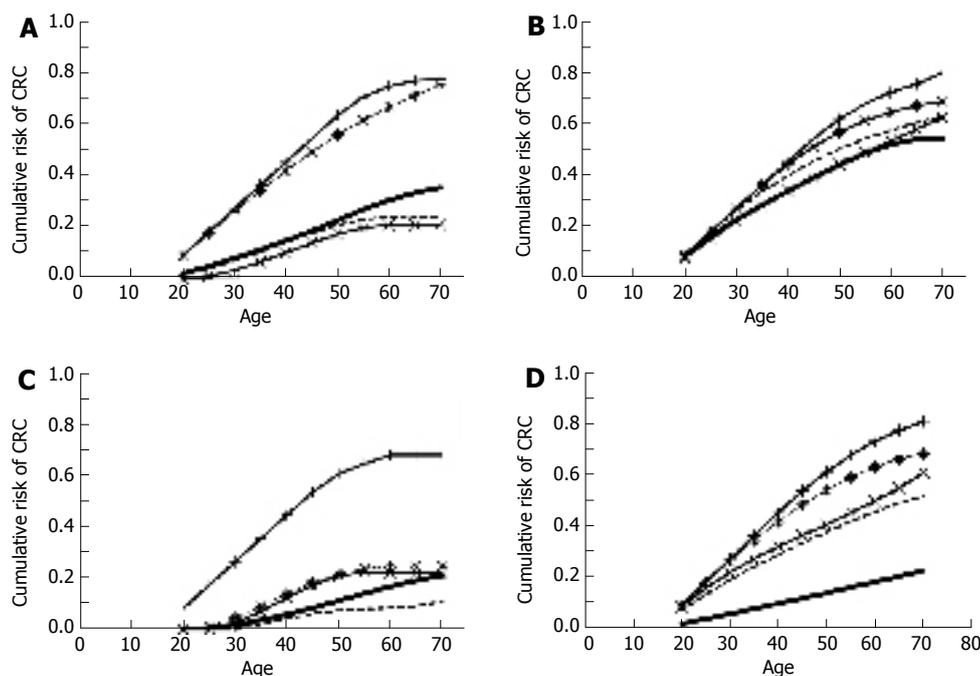


Figure 5 Probability of developing CRC (cumulative lifetime risk) based on a model containing pack years for (A) females with hMLH1 mutation ($P < 0.01$), (B) males with hMLH1 mutation ($P < 0.05$), (C) females with hMLH1 mutation ($P < 0.01$), and (D) males with hMSH2 mutation ($P < 0.01$). The P values show the probability of failing to reject the null hypothesis that the data originate from identical populations. The number of pack years is as follows: - = 0, -x = 10, --- = 20, -·- = 30 and -+ = 40.

risk of developing CRC as a function of pack years. For female smokers with a hMLH1 mutation (Figure 5A), the CRC risk was only increased after 30-pack years ($P < 0.01$), whereas for males (Figure 5B) the risk did not significantly increase until 40-pack years ($P < 0.05$). Similarly, females with a hMSH2 (Figure 5C) only demonstrated a smoking effect at 40-pack years ($P < 0.01$). Conversely, males with a hMSH2 mutation (Figure 5D) had an increased risk of developing CRC in a dose-dependent fashion in response to the number of cigarettes smoked over their lifetime ($P < 0.01$).

DISCUSSION

We have demonstrated herein that by using a fuzzy modeling approach, we could quantitatively predict the effect of environmental factors on risk of developing CRC in subjects who harbor a germline mutation for HNPCC. Importantly, we could calculate estimates for the impact of modifiable risk factors (i.e. smoking) on the occurrence of CRC in these high-risk patients and individualize the risk estimates by accounting for other major factors on the phenotypic variability in HNPCC patients: the mutated gene (hMLH1 versus hMSH2) and gender. Thus, we believe that these results may be a useful tool in patient counseling by providing concrete estimates of the impact of risk factor modification.

Our observations regarding the gene-environmental joint effects were made possible by the remarkable resource represented by the Creighton Hereditary Cancer Center Registry. Although it is one of the oldest and largest HNPCC registries in the world, a conventional statistical approach to this dataset is not powerful enough to detect the gene-environment joint effect and the dose-response of smoking and CRC^[15], because the relatively small subgroup size markedly reduces statistical power in conventional (e.g. Cox proportional hazard modeling) statistical approaches. One approach to mitigate these

concerns is to increase the size of groups (e.g. to evaluate effect of age by increasing 40-59, 60-79, *etc.*). However, such large groups have clear disadvantages. For instance, a 41-year old individual and a 58-year old individual may be quite biologically/ clinically different and yet are in the same stratum. Fuzzy modeling enables partial membership functions. For instance, a 43-year old individual may be considered to be 80% in the 40-50 group and 20% belonging to the 30-39 category, whereas a 59-year old individual may be 65% in the 50-59 group and 35% within the 60-70 group. Thus, fuzzy modeling allows us to account for the heterogeneity, i.e., “shades of gray” that is a hallmark of clinical medicine.

There are several lines of evidence that support the biological validity of our findings with fuzzy modeling. Cigarette use is an important risk factor for CRC, and 12% of all CRC deaths are attributed to smoking^[16]. Many studies indicate that cigarette smoking can increase the incidence of colon cancers by approximately two fold, however there are numerous contradictory reports^[17-19]. These discordant data have been clarified by the demonstration that cigarette smoking may selectively increase the risk for DNA mismatch repair^[20]. This may be related to the observation that smoking preferentially promotes microsatellite unstable (MSI-high) tumors. The molecular pathway is also seen in Lynch syndrome tumors^[19]. For instance, Yang *et al.*^[21] have recently reported that cigarette smoking increases the risk of developing MMR-deficient tumors by 3.1-fold. Additionally, Slattery *et al.*^[22] demonstrated that smoking 20 cigarettes per day increases the risk of MMR-deficient tumors by 1.6-fold in men (95% CI = 1.0-2.5) and 2.2-fold in women (95% CI = 1.4-3.5). Furthermore, they have documented a dose-dependent relationship between smoking and colorectal cancer^[22]. This dose-dependence underscores the plausibility of the cigarette-induced CRC risk. While we were able to discern an effect of smoking in our previous

study in HNPCC- cigarette smoking data set with Cox proportional hazard modeling, the lack of dose response raises concerns about the validity of the findings^[15]. Using fuzzy modeling we demonstrated a much clearer relationship. For instance, our dose response model predicts that female smokers with a hMLH1 mutation who have at least 30-pack years of smoking will have an increase in the lifetime risk of CRC by 2.2- fold. A 3.3-fold increase is seen for females with an hMSH2 mutation after 40 pack years. Males with a hMSH2 mutation have a more linear increase in their lifetime risk of developing CRC as a function of pack years.

This ability to quantitate an individual's risk is of paramount clinical importance due to the variability in CRC presentation that is characteristic of HNPCC. For instance, some members of a kindred may develop CRC at age 25 and 65 while other members may never develop it. Given this heterogeneity, "one size fits all" approach to management (the current state of the art) is clearly inadequate. Indeed, previous attempts to determine the optimal cancer prevention strategy (prophylactic colectomy versus colonoscopic surveillance) have failed to conclusively demonstrate the superiority of any single approach^[7]. Even determining the best colonoscopic intervals is unclear. While our group recommends annual colonoscopy starting at age 25^[23], a large number of negative examinations are expensive and have potential complications and may lead to patient complacency. Increasing surveillance intervals is fraught with danger given both the rapid adenoma to carcinoma transition and also the flat nature of the lesions, leading to a higher possibility of lesions being missed on colonoscopy^[23]. The consequences of inadequate screening are underscored by the report of Jarvinen and colleagues^[24], who noted that over a 15-year observation period, 8.4% of HNPCC patients who did not undergo screening would die of CRC whereas none of those who were in a screening program can succumb to this malignancy. Indeed, in mutation positive subjects, development of CRC occur in 42% of the non-screened but only 18% in patients receiving screening ($P < 0.02$)^[24]. Thus, implementation and adherence of a screening regimen are critical in protecting these high-risk patients against CRC.

It needs to be emphasized that the ability of fuzzy modeling to quantitate risk may be of considerable importance in counseling patients. For instance, being able to tell patients that their risk of CRC more than doubles with smoking may be more tangible than stating that smoking is detrimental to ones health, thereby providing a greater impetus for behavior modification. By accurately delineating risk, patients will be able to concretely identify modifiable risk factors and ascertain the impact of their lifestyle changes, thus providing positive reinforcement. In this regard, Halpert and colleagues^[25] noted that genetic testing of HNPCC patients may have a profound effect upon motivation for cancer prevention strategies such as colonoscopy. Improved adherence with CRC screening regimens from genetic testing and counseling is also documented by Hadley and associates^[26]. The malleability of CRC prevention behaviors in HNPCC patients is further highlighted by Adams and colleagues^[27] who

documented the effect of socio-economic considerations on age of resection of CRC in HNPCC patients. Thus, we believe that added information obtained by fuzzy modeling may have a dramatic effect on patient behavior and thus outcomes. There are previous demonstrations of the efficacy of fuzzy logic to cancer risk stratification. Fuzzy logic has been used with impressive success to improve the sensitivity of tumor markers in diagnosing cancer^[11-13].

There are several limitations of this report that need to be acknowledged. As any modeling, the accuracy of the results is dependent on quality of the data inputted. Many of our patients did not have data to quantitate pack-years. Bias in the database due to patient/family report is possible (e.g. having cancer may influence recollection of tobacco use history). Since tobacco use is not a "standard" risk-factor for CRC, we do not think this will impact the results. With smoking, there is always concern about confounding from "competing causes of mortality"^[28]. However we have recently shown that this effect is negligible for the smoking-CRC effect^[29]. Finally, while our modeling accurately reflects our database, the algorithms need to be validated in other databases.

In conclusion, a fuzzy modeling approach represents a promising means of predicting the phenotypic heterogeneity in colorectal cancer presentation in HNPCC mutation carriers. The methodology may be an important tool in unraveling the gene-environment joint effects in hereditary cancer syndromes. Furthermore, this may serve as the basis for future paradigms that determine individualized cancer prevention strategies in subjects harboring an inherited risk.

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VIRAL HEPATITIS

Inhibition of hepatitis B virus replication by APOBEC3G *in vitro* and *in vivo*

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Abstract

AIM: To investigate the effect of APOBEC3G mediated antiviral activity against hepatitis B virus (HBV) in cell cultures and replication competent HBV vector-based mouse model.

METHODS: The mammalian hepatoma cells Huh7 and HepG2 were cotransfected with various amounts of CMV-driven expression vector encoding APOBEC3G and replication competent 1.3 fold over-length HBV. Levels of HBsAg and HBeAg in the media of the transfected cells were determined by ELISA. The expression of HBcAg in transfected cells was detected by western blot. HBV DNA and RNA from intracellular core particles were examined by Northern and Southern blot analyses. To assess activity of the APOBEC3G *in vivo*, an HBV vector-based model was used in which APOBEC3G and the HBV vector were co-delivered *via* high-volume tail vein injection. Levels of HBsAg and HBV DNA in the sera of mice as well as HBV core-associated RNA in the liver of mice were determined by ELISA and quantitative PCR analysis respectively.

RESULTS: There was a dose dependent decrease in

the levels of intracellular core-associated HBV DNA and extracellular production of HBsAg and HBeAg. The levels of intracellular core-associated viral RNA also decreased, but the expression of HBcAg in transfected cells showed almost no change. Consistent with *in vitro* results, levels of HBsAg in the sera of mice were dramatically decreased. More than 1.5 log₁₀ decrease in levels of serum HBV DNA and liver HBV RNA were observed in the APOBEC3G-treated groups compared with the control groups.

CONCLUSION: These findings indicate that APOBEC3G could suppress HBV replication and antigen expression both *in vivo* and *in vitro*, promising an advance in treatment of HBV infection.

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Key words: APOBEC3G; Hepatitis B virus; Antiviral therapy

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INTRODUCTION

Hepatitis B virus (HBV) infection is a major cause of liver disease worldwide, ranging from acute and chronic hepatitis to cirrhosis and hepatocellular carcinoma^[1]. HBV replication involves reverse-transcription of a pregenomic RNA (pgRNA) intermediate inside nucleocapsids, which are formed by 180 or 240 core protein subunits. Inside the capsid, the viral polymerase converts pgRNA into minus-strand DNA, which in turn is completed to a double-stranded, relaxed circular DNA molecule^[2,3]. This life cycle places HBV into the large family of retroelements, which all require reverse-transcription of an RNA intermediate.

Recently, a cellular defense mechanism targeting a wide range of retroviruses was identified. It was shown that the propagation of HIV-1 strains lacking the accessory protein Vif is suppressed in nonpermissive cells due to expression of the cytidine deaminase APOBEC3G^[4,5]. Further studies revealed that A3G induces massive C→U deamination of single stranded retroviral DNA, resulting

in DNA degradation or lethal G→A hypermutation^[6-8]. In addition, APOBEC3F (another cytidine deaminase of APOBEC family) is also a potent retroviral restrictor, but its activity, unlike that of APOBEC3G, is partially resistant to HIV-1 Vif and results in retroviral hypermutation. Moreover, APOBEC3F and APOBEC3G appear to be coordinately expressed in a wide range of human tissues and are independently able to inhibit retroviral infection. Thus, APOBEC3F and APOBEC3G are likely to function alongside one another in the provision of an innate immune defense, with APOBEC3F functioning as the major contributor to HIV-1 hypermutation *in vivo*^[9]. Interestingly, APOBEC3G can also interfere with the HBV life cycle in cotransfected cells^[10,11]. Surprisingly, however, reduced levels of encapsidated viral pgRNA rather than extensive editing were found to be the major contributing factor^[10]. APOBEC3G-mediated editing did occur but was only detected in a minority of clones produced in transfected HepG2 hepatoma cells^[11].

In the present study, we performed a detailed analysis of the inhibition effect of APOBEC3G on hepatitis B virus replication in cell culture and in an HBV vector-based mouse model.

MATERIALS AND METHODS

Plasmid constructs

To construct expression vectors coding for human APOBEC3G (pXFA3G), total RNAs were extracted from peripheral blood mononuclear cells (PBMCs). RT-PCR amplification of human APOBEC3G sequence used forward primer 5'-CGGAATTC AAGCCTCACTT CAGAAACAC-3' and reverse primer 5'-CGAAGCTT TCTGCCTTCCTTAGAGACT G-3'. The PCR product was cloned into EcoRI/HindIII restriction sites of the CMV-driven expression vector fused with a hemagglutinin fusion epitope tag at its carboxy terminal end (pXF3H). Replication competent wild-type HBV 1.3 fold over-length plasmid (subtype, ayw) has been constructed previously in our laboratory.

Cell culture, transfection and harvesting

HuH-7 cells were grown at 37°C with 50 mL/L CO₂ in Dulbecco's modified Eagle medium, supplemented with 10% fetal calf serum (Invitrogen, CA, USA). The cells were plated at a density of 4.5×10^5 cells per well in 6-well plates 18 h prior to transfection. Transfection of cells was performed with lipofectamine 2000 (Invitrogen, CA, USA) following the user guidelines. On d 3 after transfection, the cells were removed from the culture dish by treatment with trypsin-EDTA, resuspended in culture medium, washed with phosphate-buffered saline, pelleted, and resuspended in 1 mL chilled lysis buffer (140 mmol/L NaCl, 1.5 mmol/L MgCl₂, 50 mmol/L Tris-HCl [pH 8.0]) containing 0.5% Nonidet P-40. Nuclei were removed *via* centrifugation for 5 min at 2000 r/min in an Eppendorf centrifuge, and the supernatant was cleared of cell debris by centrifugation for another 5 min at 14000 r/min.

HBV vector-based mouse model

For the *in vivo* experiments, 6 to 8 wk-old female BALB/c

mice were used. A total of 18 mice were randomly divided into 3 groups (6 mice in each). Replication competent pHBV1.3 (10 µg) and APOBEC3G expression vectors pXFA3G (10 µg) or pXF3H (10 µg) control plasmid DNA were co-injected into the tail vein of mice in a volume of Ringer's injection equivalent to 9% of the mouse body weight^[12,13]; the total volume was delivered within 5 s. The mouse sera were collected at indicated time after hydrodynamic injection, and secreted HBsAg levels and HBV DNA content were measured. All mouse experiments were carried out according to the guidelines established by the Institutional Animal Care and Use Committee at the Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China.

HBsAg and HBeAg assays and western blot analysis

Levels of HBsAg and HBeAg in the media of the transfected cells, and in the sera (1:100 diluted) of the treated mice were determined using ELISA (Shanghai Shiyeh Kehua Company, China). For western blot analysis, cytoplasmic lysates were incubated with 1 volume 2 x loading buffer containing 10% beta-mercaptoethanol for 10 min at 95°C before loading onto a 12.5% SDS-PAGE. Proteins were transferred onto nitrocellulose membrane *via* electroblotting. The membranes were incubated with HBV core specific rabbit antiserum (Santa Cruz, USA) or with anti-hemagglutinin fusion epitope monoclonal antibody (Santa Cruz, USA) followed by horseradish peroxidase-conjugated mouse anti-rabbit antibody. Proteins were visualized *via* enhanced chemiluminescence (Roche, Germany).

HBV DNA purification and analysis

The method for purification of cytoplasmic core-associated HBV DNA was adapted from Pugh *et al.*^[14]. Briefly, Huh7 cells were disrupted in lysis buffer (100 mmol/L Tris-HCl pH 8.0, 0.2% NP-40). The cell lysate was clarified by centrifugation at 13000 r/min for 1 min to pellet nuclei and insoluble material. The supernatant was adjusted to 6 mmol/L MgOAc₂ and incubated for 2 h at 37°C with 200 µg/mL of DNase I and 100 µg/mL of RNase A. Following digestion, the lysate was centrifuged for 1 min at 13000 r/min. The supernatant was incubated for 1 h at 55°C after addition of 10 mmol/L EDTA, 1% SDS, 100 mmol/L NaCl and 200 µg/mL of proteinase K. Finally, the sample was extracted with phenol: chloroform. The DNA was ethanol precipitated, resuspended in TE pH 8 (10 mmol/L Tris-HCl pH 7.5, 1 mmol/L EDTA) and digested with 100 ng/µL of RNase A for 30 min at 37°C. Purified DNA was subjected to Southern blot analysis. DNA samples were loaded onto 1.3% agarose gels, blotted onto nylon membranes, and probed with a Dig-labeled full-length HBV genome in EasyHyb hybridization solution (Roche, Germany).

For quantitative PCR, 100 µL mouse serum was adjusted to 6 mmol/L MgOAc₂ and incubated for 2 h at 37°C with 200 µg/mL of DNase I and 100 µg/mL of RNase A. Following proteinase K digestion, the sample was extracted with phenol: chloroform. HBV DNA levels were analyzed with the Light Cycle real-time PCR system (Roche, Germany), and primer sequences as follows: 5'-TCACAATACCGCAGAGTC-3' (nt231-248, forward), 5'

- AGCAACAGGAGGGATACA-3' (nt569-552, reverse). The pHBV1.0 vector containing the full-length HBV genome was used as a standard curve to calculate HBV copies per milliliter of serum.

RNA isolation and analysis

For Northern blot analysis, total RNA was extracted from transfected cells with TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. Twenty microgram RNA was separated by electrophoresis on a 1% agarose gel and transferred onto nylon membrane, hybridized with a DIG-labeled DNA fragment covering the full-length HBV sequence. The probe was generated with a PCR DIG probe synthesis kit (Roche, Germany). For Northern blot analysis, RNA samples were resolved on MOPS-buffered 1.2% agarose gels containing 1.8% formaldehyde followed by capillary transfer onto nylon membranes and hybridization with a ³²P-labeled full-length HBV probe.

For quantitative RT-PCR, approximately 20 mg of liver tissue was obtained from mice for total RNA extraction with RNeasy total RNA kit (QIAGEN, Germany), according to the manufacturer's protocol. cDNA was synthesized from 2 µg of total RNA with oligo(dT)₁₅ primer in a total volume of 20 µL. Quantitative RT-PCR was performed with the Light Cycle real-time PCR system (Roche, Germany). The PCR primers are as follows, GAPDH: 5'-GTTGTCTCCTGCGACTTCA-3' (forward), 5'-GGTGGTCCAGGGTTTCTTA-3' (reverse); HBV: 5'-TCACAATACCGCAGAGTC-3' (nt231-248, forward), 5'-AGCAACAGGAGGGATACA-3' (nt569-552, reverse). A standard curve was constructed by the simultaneous amplification of serial dilutions of the expression plasmid encoding HBV used as templates. Target cDNAs were normalized to the endogenous RNA levels of the GAPDH. Quantitative amplification was carried out using the SYBR Green kit (Invitrogen, USA). Gene expression was determined using the relative quantification: $\Delta\Delta C_T = (C_{T\text{Target}} - C_{T\text{GAPDH}})_{\text{Test}} - (C_{T\text{Target}} - C_{T\text{GAPDH}})_{\text{Control}}$. C_T is the fractional cycle number that reaches a fixed threshold, C_{Test} is the test of HBV, and C_{Control} is the reference control (RNA from control group). ΔC_T is the difference between gene expression in treated cells and reference control cells. The fold increase was calculated using $2^{-\Delta\Delta C_T}$ [14].

RESULTS

Inhibitory effect of APOBEC3G on HBV DNA replication in cell culture

The mammalian hepatoma cells HuH-7 and HepG2 were co-transfected with replication-competent over-length 1.3 fold HBV and various amounts of a CMV-driven expression vector encoding APOBEC3G. In order to determine the efficiency of transfection, Western blot with polyclonal anti-HA and anti-HBV core antibody was used to analyze the expression of APOBEC3G and HBV core protein in co-transfected Huh7 cells. Three days after transfection, core-associated viral DNA was prepared from nuclease-treated cytoplasmic lysates and analyzed with Southern blotting. As shown in Figures 1 and 2, APOBEC3G and HBV core protein were expressed

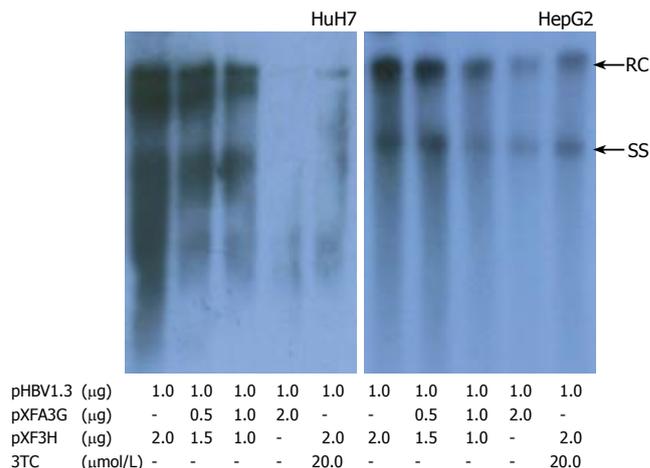


Figure 1 Effect of APOBEC3G on HBV replication in cotransfected hepatoma cells. Cells were treated with lipofectamine 2000 reagents, and viral replicative DNA intermediates were analyzed with southern blotting 3 d after transfection. Numbers at the end of the lines indicate the amount of transfected plasmid DNA in micrograms. HBV, hepatitis B virus; RC, relaxed circular DNA; SS, single-stranded DNA.

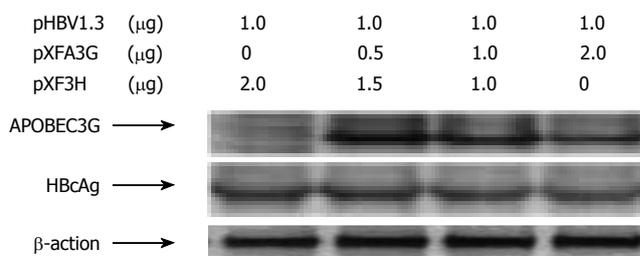


Figure 2 Western blot analysis of cytoplasmic extracts from Huh7 cells cotransfected with indicated plasmids.

in co-transfected Huh7 cells. However, APOBEC3G reduced the level of replicative HBV intermediates in a dose-dependent manner. This inhibitory effect was also observed in HepG2 cell lines.

Inhibition of HBV gene expression by APOBEC3G in cell culture

To evaluate the effects of APOBEC3G on HBV gene expression, Huh7 cells were co-transfected with replication competent over-length HBV (pHBV1.3) and various amounts of CMV-driven expression vector encoding APOBEC3G 3 d after transfection. HBsAg and HBeAg levels were determined by ELISA. As shown in Figure 3, there was also a dose dependent decrease in the levels of extracellular production of HBsAg and HBeAg. However, western blot with polyclonal anti-HBV core antibody indicates that the levels of core protein were unaffected by APOBEC3G (Figure 2). In order to examine the effect of APOBEC3G on HBV RNA levels in co-transfected Huh7 cells, total cellular RNA derived from co-transfected Huh7 cells was hybridized with Dig-labeled probe. Northern blot indicated that the RNA levels of 3.5 kb, 2.4/2.1 kb in co-transfected Huh7 cells were significantly suppressed by APOBEC3G (Figure 4).

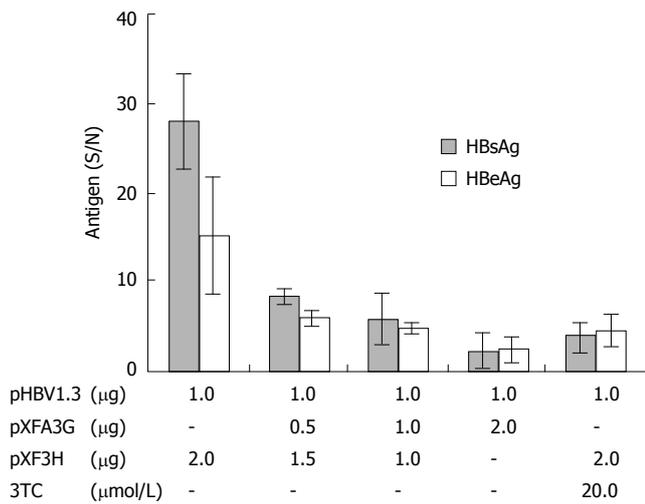


Figure 3 Effect of APOBEC3G on HBsAg and HBeAg secretion in the media of cotransfected Huh7 cells 3 d after transfection. HBsAg and HBeAg levels (S/N) were significantly reduced (error bar indicate standard error). Numbers at the end of the lines indicate the amount of transfected plasmid DNA in micrograms.

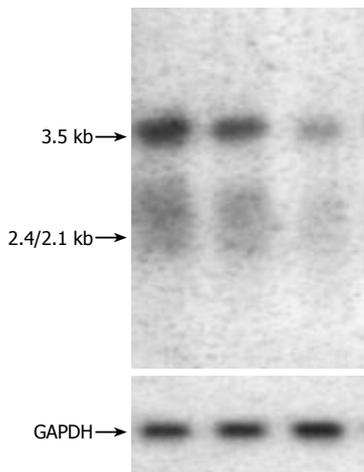


Figure 4 Effect of APOBEC3G on HBV RNA expression. Northern blot analysis of HBV transcripts from Huh7 cells co-transfected with indicated plasmids. The same blot was hybridized with a glyceraldehyde phosphate dehydrogenase (GAPDH) DNA probe as an internal control.

pHBV1.3 (μg)	pXF3G-HA (μg)	pXF3H (μg)
1.0	-	2.0
1.0	1.0	1.0
1.0	2.0	-

Inhibition of HBV replication and gene expression by APOBEC3G in mice

To examine whether APOBEC3G could also display HBV inhibition *in vivo*, replication-competent HBV plasmid (pHBV1.3) was co-transfected with pXFA3G or pXF3H(control plasmid) to mouse liver by hydrodynamic injection. As shown previously, hydrodynamic injection of pHBV1.3 into mouse resulted in HBV replication in the liver and secretion of viral antigens to the serum. Consistent with *in vitro* experiments, serum HBsAg and viral DNA in mice which received pHBV1.3 was suppressed dramatically at different time points by APOBEC3G compared with mice without APOBEC3G treatment (Figures 5 and 6). Real time RT-PCR quantification of core-associated HBV RNA in the liver of mice 3 d after injection with APOBEC3G expression plasmid was decreased about 50 times than that of control group (Figure 7).

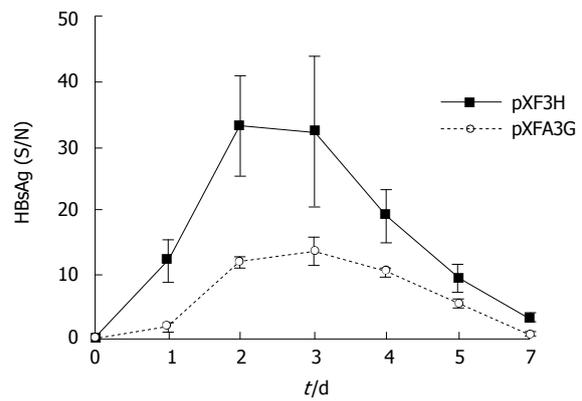


Figure 5 Serum HBsAg levels in APOBEC3G treated mice. HBsAg levels(S/N) in the sera of BALB/c mice were significantly reduced after treatment with the APOBEC3G expression plasmids (error bar indicate standard error) in the indicated time points, *n* = 6 per treatment group.

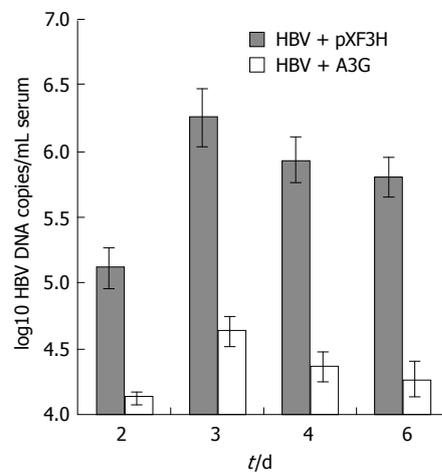


Figure 6 Real time PCR quantification of HBV DNA in the sera of mice treated with APOBEC3G expression plasmid. BALB/c mice were coinjected with pHBV1.3 vector (10 μg) and APOBEC3G expression plasmid (10 μg) 3 d after injection. HBV DNA levels (mean ± SD) of sera were determined by quantitative PCR, *n* = 6 per treatment group.

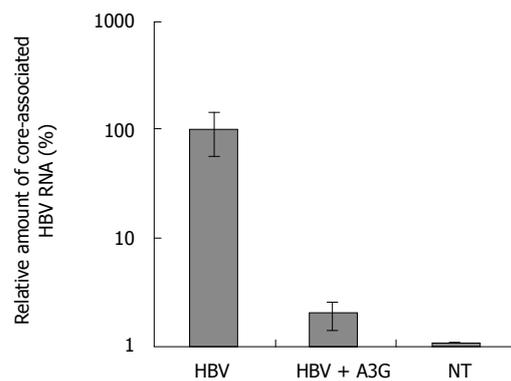


Figure 7 Real time PCR quantification of core-associated HBV RNA in the liver of mice treated with indicated plasmids. BALB/c mice were coinjected with pHBV1.3 vector (10 μg) and APOBEC3G expression plasmid (10 μg) 3 d after injection. The levels of liver HBV RNA were determined by quantitative RT-PCR, normalized to GAPDH mRNA and are reported as a ratio of HBV mRNA/GAPDH mRNA (mean ± SD). *n* = 6 per treatment group.

DISCUSSION

This study explored the effects of human APOBEC3G suppressing HBV DNA production in mammalian hepatoma cells as well as in HBV vector-based mouse model. Our data confirm and extend recent results by

Turelli *et al*^[10] who reported A3G-mediated inhibition of HBV DNA production in human HuH-7 hepatoma cells. Previous studies suggested that G-to-A hypermutation can influence HBV pathogenesis. Specific G-to-A changes yield HBeAg-negative HBV variants, often isolated from patients with acute fulminant hepatitis, as well as HBV vaccine escape mutants^[17,18]. However, there is no evidence so far that APOBEC-induced lethal hypermutation represents an important innate defense mechanism down regulating hepadnavirus production. APOBEC3G-mediated editing did occur but was only detected in a minority of clones produced in transfected HepG2 hepatoma cells^[10]. Recently, Rosler *et al*^[19] demonstrated that APOBEC3G rendered HBV core protein associated full-length pregenomic RNA nuclease-sensitive. APOBEC3G-mediated editing of nucleic acids does not seem to represent an effective innate defense mechanism for hepadnaviruses.

During the course of an acute infection, HBV DNA clearance apparently occurs through noncytopathic mechanisms in which IFN- γ and TNF- α play an important role^[20,21]. APOBEC3G might participate in this type of antiviral response. Although APOBEC3G is not normally expressed in the liver^[10,22], recent study shows that APOBEC3G is induced by interferon- α stimulation in human hepatocytes and human primary monocyte-derived macrophages^[16,23]. It is possibly induced by interferon in the course of HBV infection. Several studies have shown that IFNs inhibit HBV replication *in vitro* in human hepatoma cells and HBV transgenic mice *in vivo*^[24,26]. Currently, IFN- α is approved for treatments of chronic hepatitis B. We speculate that APOBEC3G might be responsible for the anti-HBV action of IFNs in hepatic inflammation. Further analyses will be necessary to determine whether APOBEC3G play roles in the host innate defense against hepatitis viruses *in vivo*.

The hydrodynamic delivery of nucleic acids in the mouse was described by Liu *et al*^[11], who showed that the vast majority of the injected nucleic acid was delivered to the liver by this technique. Yang *et al*^[27] first demonstrated that hydrodynamic injection of a replication-competent HBV vector resulted in high levels of HBV replication in the livers of injected mice. In the vector-based model, HBV replicates in the liver of immunocompetent mice for 7 to 10 d, resulting in detectable levels of HBV RNA and antigens in the liver and HBV DNA and antigens in the serum. Several reports have documented the use of HBV vector model to examine the *in vivo* activity of co-HDI administered HBV-targeted unmodified siRNAs^[28,29] or vector-expressed short hairpin RNAs in silencing HBV gene expression^[30]. Most notably, we have demonstrated *in vivo* activity of APOBEC3G expression vector *via* standard intravenous administration. This is the first demonstration of APOBEC3G *in vivo* activity in a hepatitis animal model with a clinically viable route of administration. Although we have seen a more than 1.5log10 reduction of serum HBV levels with APOBEC3G in an HBV mouse model, however, the potential contribution of toxicity from the APOBEC3G has not been ruled out and needs to be investigated further.

Our work demonstrates APOBEC3G effectively inhibits HBV replication in culture cells and mammalian liver, suggesting that such an approach could be useful in the treatment of HBV infection. However, whether suppression of viral replication by APOBEC3G plays a role during natural HBV infection seems speculative at present, because expression of APOBEC3G in human liver tissue has not yet been shown. Nevertheless, a better understanding of the mechanisms of APOBEC3G action may help identify new therapeutic strategies against chronic hepatitis B.

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BASIC RESEARCH

Mechanism for Src activation by the CCK2 receptor: Pathophysiological functions of this receptor in pancreas

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Abstract

AIM: To investigate *in vivo*, whether CCK2 receptors (CCK2R) regulate proteins known to play a crucial role in cell proliferation and cancer development and analyse *in vitro* the molecular mechanisms that lead to Src activation; in particular, to identify the domains within the CCK2R sequence that are implicated in this activation.

METHODS: The expression and activation of Src and ERK were studied *in vivo* using immunofluorescence and western-blot techniques. We used pancreatic tissues derived from wild type or Elas-CCK2 mice that expressed the CCK2R in pancreatic acini, displayed an increased pancreatic growth and developed preneoplastic lesions. The pancreatic tumor cell line AR4-2J expressing the endogenous CCK2R or COS-7 cells transiently transfected with wild type or mutant CCK2R were used as *in vitro* models to study the mechanism of Src activation. Src activation was measured by *in vitro* kinase assays, ERK activation by western blot using anti-phospho-ERK antibodies and the involvement of Src in gastrin-induced cell proliferation by MTT test.

RESULTS: We showed *in vivo* that the targeted CCK2R expression in the pancreas of Elas-CCK2 mice, led to the activation of Src and the ERK pathway. Src was activated upstream of the ERK pathway by the CCK2R in pancreatic tumoral cells and contributed to the proliferative effects mediated by this receptor. *In vitro* results demonstrated

that activation of the Src/ERK pathway by the CCK2R required the NPXXY motif, located within the CCK2R sequence at the end of the 7th transmembrane domain, and suggested the putative role of Gq in this mechanism.

CONCLUSION: Deregulation of the Src/ERK pathway by the CCK2R might represent an early step that contributes to cell proliferation, formation of preneoplastic lesions and pancreatic tumor development.

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Key words: Gastrin; Src; Pancreas; CCK2 receptor

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INTRODUCTION

The CCK2 receptor (CCK2R or CCKBR) is a G protein-coupled receptor, mainly coupled to Gq proteins^[1]. Initially implicated in gastrin-mediated acid secretion CCK2R is now recognized to mediate mitogenic and anti-apoptotic effects on gastrointestinal and pancreatic cells^[2]. In the transgenic mice, Elas-CCK2, CCK2R expression has been targeted in pancreatic acinar cells using transcriptional elements of the elastase-1 promoter^[3]. Using this model, we have recently reported an increase in pancreatic growth as well as an acinar to ductal transdifferentiation, postulated to be a preneoplastic step in pancreatic carcinogenesis that precedes the development of tumours^[3]. Similar observations have been reported in two other transgenic models overexpressing TGF- α or a Kras mutant in exocrine pancreas^[4,5].

Src family kinases are non receptor protein tyrosine kinases that mediate a wide variety of biological effects including cell survival, adhesion and proliferation. They are activated by many growth factor receptors. In addition, p60-Src is well established as an oncogene, and overexpression of Src tyrosine kinase has been described in human pancreatic adenocarcinomas^[6]. Recently, Src inhibition by

AZM475271 demonstrated significant antitumorigenic and anti-metastatic activity in an orthotopic nude mouse model for human pancreatic cancer^[7]. However to our knowledge, there is currently little or no information regarding the deregulation of Src kinases in early stages of pancreatic carcinogenesis.

It is well established that numerous G protein-coupled receptors activate Src family kinases. However, very few publications have described the molecular mechanisms involved in Src activation by this receptor family. Molecular mechanisms of Src activation by CCK2R as well as the role of this kinase and related signalling pathways in the pathophysiological functions of CCK2R *in vivo* remain largely unknown. This study had two main aims: First, to investigate *in vivo*, using the Elas-CCK2 mouse model, whether CCK2R is involved in the regulation of proteins known to play a crucial role in cell proliferation and cancer development; Second, to analyse *in vitro* the molecular mechanisms that lead to Src activation and in particular, to identify the domains within the CCK2R sequence implicated in this activation.

MATERIALS AND METHODS

Animals

Homozygous Elas-CCK2 mice used in this study have been previously described^[3]. At least 3 homozygous Elas-CCK2 mice in a B6SJLF1 background and 3 corresponding control littermate mice were used (six months old). Mice were reared in routine animal facility of the IFR31 and maintained on a 12:12 h light-dark cycle. All the experiments were performed during the daytime. All procedures were approved by the IFR31 animal facility care committee.

Antibodies and materials

GAPDH was provided by Chemicon; phospho-tyr418-Src (IF and WB) by Biosource; ERK, Src (IF and WB) by Santa Cruz Biotechnology; phospho-ERK (IF and WB) by Cell Signaling; SRC (IP) by Oncogene Research Product; PP2, GP2A by Calbiochem.

Immunofluorescence staining

Mice were killed by decapitation and the pancreas was excised, fixed in Bouin's solution and embedded in paraffin using standard techniques. Immunofluorescence staining was performed as previously described^[8]. The detection was done using secondary antibodies coupled to Alexa Fluor 488. Slides were analyzed on a Nikon E400 microscope with a Sony DXC 950 camera and Visiolab 2000 software. For semi-quantitative comparisons, identical volumes of antibody were used for all samples and identical exposure times taken.

Western-blot analysis

Western-blot analyses were performed on dispersed acini from mouse pancreas prepared as previously described^[9], and on cell lysates or immunoprecipitates from AR4-2J or COS7 cells stimulated or not with gastrin. Fractions, containing identical levels of proteins, were separated

by SDS-PAGE and analyzed by western-blot with the indicated antibodies as described previously^[9].

Cell culture and proliferation assay

AR4-2J cells and COS-7 cells were grown in DMEM supplemented with 10% and 5% fetal calf serum (FCS), respectively, at 37°C in a 95% air, 5 mL/L CO₂ atmosphere. For proliferation assays, an optimal number of AR4-2J cells (4×10^4 cells) were plated in 35-mm dishes, serum-starved for 24 h, then treated for 48 h with gastrin (10 nmol/L). When indicated, cells were incubated with PP2 (10 μmol/L). Cells were counted by using a Coulter electronic counter.

Src kinase assay

After gastrin stimulation, cells were lysed and Src immunoprecipitated with specific antibodies. Kinase assays were performed and analyzed as previously described^[10]. Proteins were separated by SDS-PAGE and the gel autoradiographed.

Construction of mutant receptor cDNAs and transient transfection

Mutant CCK2R, N386A-CCK2R was previously described^[9]. Plasmids coding for wild type or mutant CCK2R (6 μg) were transiently transfected into COS-7 cells using the DEAE/dextran method as described previously^[1].

Statistical analysis

Data were expressed as mean ± SE and Student's *t* test was performed using "GraphPad Prism". *P* < 0.05 was taken as significant.

RESULTS

Src status in Elas-CCK2 mice

Src activation was analyzed by immunohistochemical methods on pancreatic sections from control and Elas-CCK2 mice of 6 month old using antibodies specific for total Src or detecting the activated form of the protein phosphorylated on tyrosine 418 (P-Src). Acinar tissues derived from Elas-CCK2 mice demonstrated higher levels of Src activation as compared to control mice (Figure 1A, upper panels). In contrast, total Src protein expression was unchanged in the two mouse models (Figure 1A, lower panels).

To confirm and quantify Src activation, western-blot analyses were performed on lysates of acinar cells isolated from pancreas of control and Elas-CCK2 mice. Src activation in Elas-CCK2 mice was significantly elevated compared to controls (Figure 1B). Thus, the expression of the CCK2R in mouse pancreatic acini induced Src activation.

The ERK pathways in Elas-CCK2 mice

Activation of the ERK pathways by gastrin has previously been described *in vitro*^[10,11]. However, there is currently no *in vivo* information about the status of the ERK pathways in gastrin signaling. Immunofluorescence analysis was per-

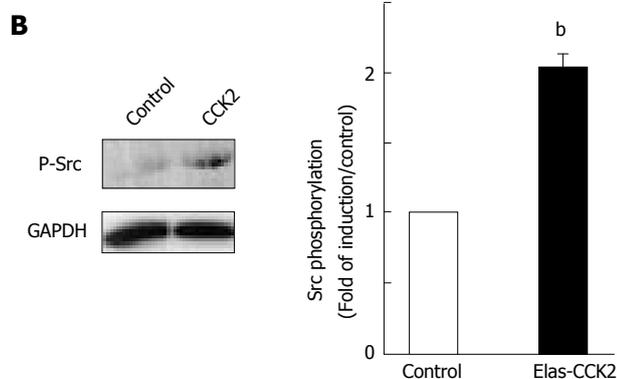
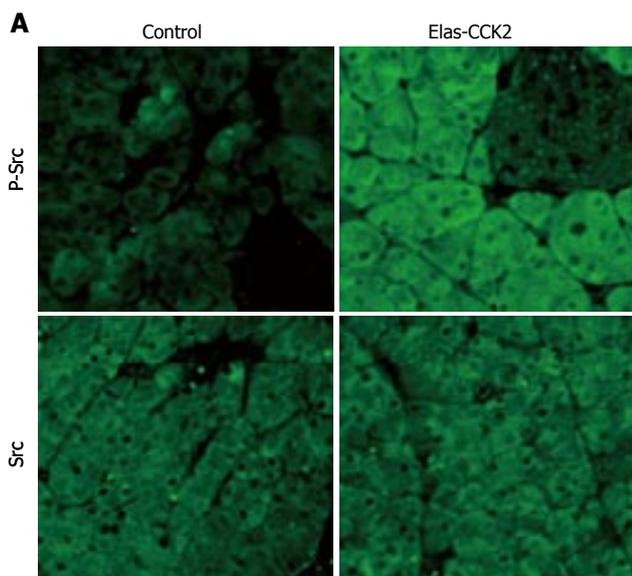


Figure 1 CCK2R expression in acini of Elas-CCK2 mice induced the activation of Src. Immunohistochemistry analysis on paraffin-embedded pancreatic tissues or western-blots on lysates from isolated acinar cells were performed using antibodies specific for total Src (SRC) or detecting the activated form of the protein, PY418-Src (P-Src) as indicated. Representative data from 3 experiments (3 different animals in each group) are shown. **A:** Original magnification: 40 X; **B:** Blots were also probed with an antibody against GAPDH to ensure equal loading of proteins. Results of western-blots quantification are presented as mean \pm SE. ^b $P < 0.01$ vs control.

formed using antibodies specific for dually phosphorylated active ERK (P-ERK). Results demonstrated an increased immunoreactivity in acinar tissue of transgenic mice as compared to control mice (Figures 2A, upper panel). In addition, pancreatic acinar tissues of Elas-CCK2 mice also showed a higher level of total ERK expression as compared to control mice (Figure 2A, lower panel). These results were confirmed by western-blots performed on lysates of acinar cells isolated from pancreas of control and Elas-CCK2 mice (Figure 2B). Overall, these results were consistent with an upregulation of the ERK pathway in the pancreas of mice expressing the CCK2R.

Src activation by CCK2R in tumour pancreatic acinar cells

The pancreatic tumour cell line AR4-2J, that exhibits an acinar phenotype, was previously shown to express endogenous CCK2R^[12]. We used this model to analyse the role of Src in the pathophysiological functions of the CCK2R and the molecular mechanisms potentially involved in Src activation by this G protein-coupled

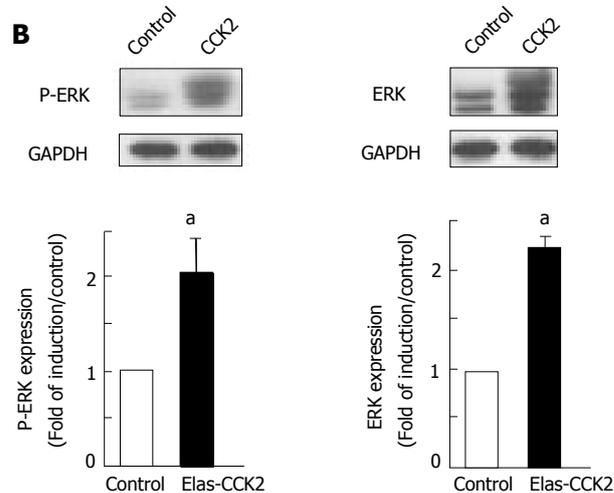
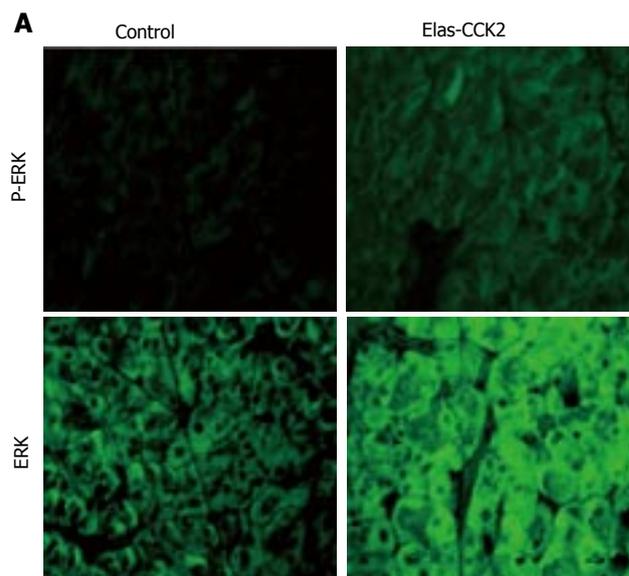


Figure 2 CCK2R expression in acini of Elas-CCK2 mice induces the activation of the ERK pathway. Immunohistochemistry analysis on paraffin-embedded pancreatic tissues or western-blots on lysates from isolated acinar cells were performed using antibodies specific for total ERK (ERK) or detecting the activated form of the protein, Phospho-ERK (P-ERK) as indicated. Representative data from 3 experiments (3 different animals in each group) are shown. **A:** Original magnification: 40 X; **B:** Blots were also probed with an antibody against GAPDH to ensure equal loading of proteins. Results of western-blots quantification are presented as mean \pm SE. ^a $P < 0.05$ vs control.

receptor.

We first confirmed that the CCK2R was able to induce Src activation. *In vitro* tyrosine-kinase assays were performed in anti-Src immunoprecipitates from cell lysates containing equal amounts of protein. We detected a rapid and significant increase in Src activation 15 s after stimulation of the CCK2R by gastrin (Figure 3A).

In order to address the role of Src family kinases in proliferation of tumour acinar cells induced by CCK2R, we measured AR4-2J proliferation in the presence or absence of the Src specific inhibitor, PP2, 48 h after gastrin stimulation. CCK2R activation by gastrin induced a significant increase of cell proliferation. Treatment of cells with PP2 totally inhibited CCK2R-induced AR4-2J proliferation (Figure 3B).

We previously reported that CCK2R associates with the α q subunit of heterotrimeric G-proteins^[1]. We therefore

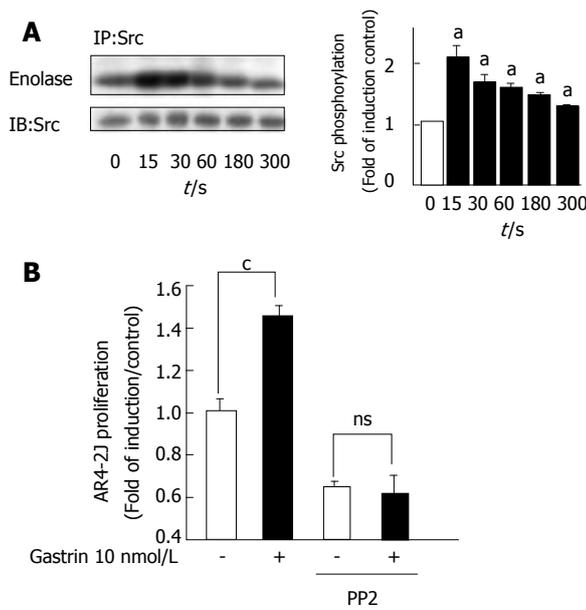


Figure 3 Role of Src in proliferation of tumour pancreatic acinar cells induced by the CCK2R. AR4-2J cells were stimulated with Gastrin (10 nmol/L) for the times indicated (A) or 15 s (B). A: Src activity was determined as described in Methods. Immunoprecipitated proteins were also analysed by western-blot using anti-Src antibodies. Results of autoradiography quantification are presented as mean ± SE. ^a*P* < 0.05 vs control. B: Serum-starved AR4-2J cells were treated with Gastrin for 48 h in the presence or absence of PP2 (10 μmol/L), and the proliferation determined as described in Methods. Data are presented as mean ± SE. ^c*P* < 0.001 vs control.

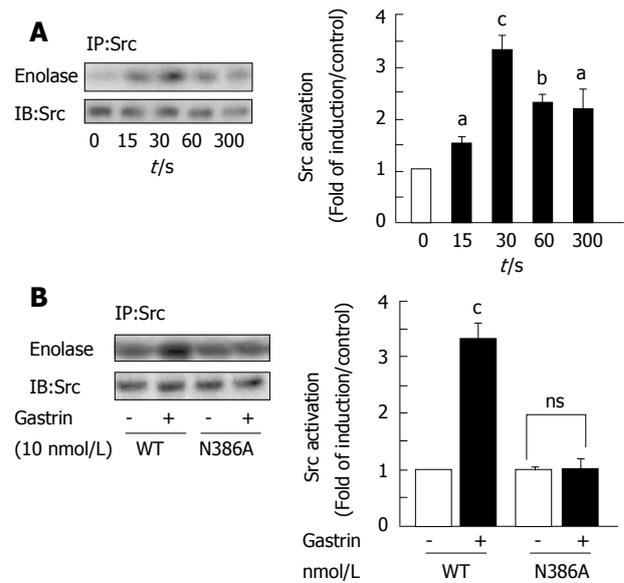


Figure 5 Involvement of the NPXXY motif in Src activation by the CCK2R. A: COS-7 cells transfected with the human CCK2R were stimulated with Gastrin (10 nmol/L) for the time indicated. Src kinase activity was determined as described in Methods. Immunoprecipitated proteins were also analysed by western-blot using anti-Src antibodies. Results of autoradiography quantification are presented as mean ± SE. ^a*P* < 0.05 vs control, ^b*P* < 0.01 vs control, ^c*P* < 0.001 vs control; B: COS-7 cells transfected with the WT or N386A mutant CCK2R were stimulated or not with Gastrin (10 nmol/L) for 30 s and Src kinase activity determined as described in Methods. Immunoprecipitated proteins were also analysed by western-blot using anti-Src antibodies. Results of autoradiography quantification are presented as mean ± SE. ^c*P* < 0.001 vs control.

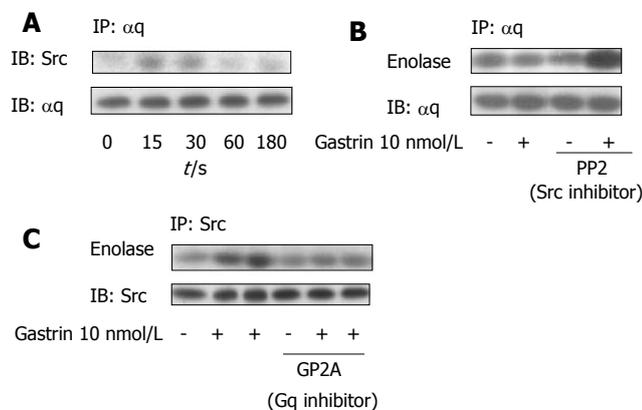


Figure 4 Src activation by the CCK2R in tumour pancreatic acinar cells (A-C). AR4-2J cells were stimulated with Gastrin (10 nmol/L) for the times indicated (A) or for 15 s (B, C). When indicated, cells were pretreated with 10 μmol/L of PP2 or GP2A for 30 min. A: Cell lysates were immunoprecipitated (IP) with an anti-αq antibody and immunoblotted (IB) with the anti-Src antibody. The blots were also probed with the antibody used for immunoprecipitation to ensure equal loading of proteins; B, C: following immunoprecipitation with an anti-αq or an anti-Src antibody, Src activity was determined as described in Methods. Immunoprecipitated proteins were also analyzed by western blot using the anti-αq or anti-Src antibodies as indicated.

tested the hypothesis that Gαq might be involved in gastrin-induced Src activation.

Src proteins were immunoprecipitated with specific antibodies and their association with Gαq was analyzed by western blot with an anti-αq antibody. An increase in the amount of Src proteins coprecipitated with Gαq was detected in response to gastrin (Figure 4A). This effect was time-dependent and the kinetic correlated with that of Src activation in this cellular model. We then

examined whether Src-like tyrosine kinase activity was in association with Gαq following gastrin stimulation. An increase in tyrosine kinase activity was detected in anti-Gαq immunoprecipitates after gastrin stimulation which was abolished when samples were treated with the specific Src inhibitor, PP2 (Figure 4B). In addition, pretreatment of the AR42J cells with a specific Gαq inhibitor, GP2A, completely blocked the activation of Src by gastrin (Figure 4C).

Mechanism of CCK2R-induced Src activation in COS-7 cells

To further investigate the molecular mechanisms involved in Src activation by the CCK2R we used COS-7 cells transiently transfected with cDNAs coding for the human wild type CCK2R (WT-CCK2R) or mutant CCK2R. We first validated this model for CCK2R-induced Src activation. Lysates from cells stimulated or not with gastrin were immunoprecipitated with anti-Src antibodies and kinase assays performed as described in methods. A rapid activation of Src (15 s), still detectable at 1 and 5 min, was found in response to Gastrin (Figure 5A). Western-blot analysis for Src protein expression revealed an equal amount of the protein in transfected cells.

Recently, we reported that the NPXXY motif (X represents any amino acid), located at the end of the 7th transmembrane domain of the CCK2R, was involved in Gq-dependent signaling pathways induced by the CCK2R^[1,9]. In particular, mutation of the asparagine (N) into alanine (A) inhibits Gq-dependent pathways such as inositol triphosphate (IP3) production. We therefore tested the in-

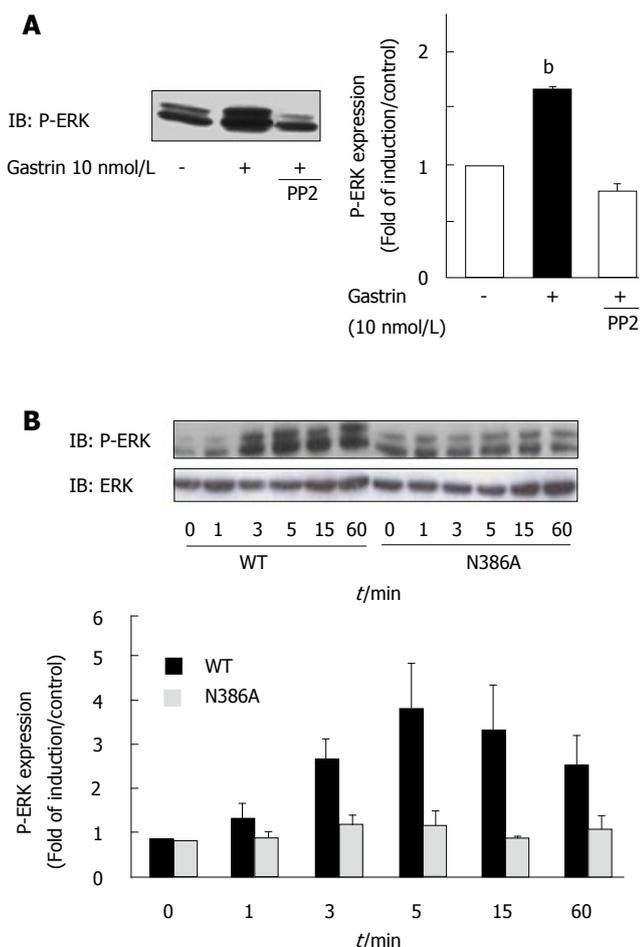


Figure 6 Involvement of the NPXXY motif in ERK activation by the CCK2R. COS-7 cells transfected with the WT or N386A mutant CCK2R were stimulated or not with Gastrin (10 nmol/L) for 5 min (A) or the time indicated (B). When indicated, cells were pretreated with the Src inhibitor PP2 (10 μ mol/L). Equal amounts of protein were analyzed by western-blot using anti-phospho-ERK antibodies. Blots were also reprobbed with antibodies directed against total ERK proteins. Results of autoradiography quantification are presented as mean \pm SE. ^b $P < 0.01$ vs control.

involvement of the NPXXY motif in Src activation by the CCK2R.

cDNAs coding for the WT receptor or the N386A mutant were transfected in COS-7 cells and Src activation was studied in response to Gastrin. Figure 5B clearly demonstrates that the CCK2R mutant cannot induce Src activation in response to Gastrin in contrast to the WT receptor.

In addition, we demonstrated that ERK activation following gastrin stimulation was completely blocked by the Src inhibitor PP2, indicating that CCK2R-induced ERK activation was totally Src-dependent in this cellular model (Figure 6A). As expected, activation of the ERK pathway was also blocked when the CCK2R was mutated on the NPXXY motif (Figure 6B). Thus, our results demonstrated the involvement of the conserved NPXXY motif within the receptor sequence in the activation of the Src/ERK pathway by the CCK2R, and suggested putative role of Gq in this mechanism.

DISCUSSION

It is well demonstrated that numerous G protein-coupled

receptors activate Src family kinases. However, very few publications have described the molecular mechanisms involved in Src activation by this receptor family. In the present study our results demonstrate that CCK2R-induced Src activation requires the NPXXY motif, and suggest a putative role of Gq in this mechanism. Indeed, this motif was previously described in Gq-dependent signalling pathways^[1,9]. In addition, we showed that Src and G α q are within one protein complex and we observed a Src-like tyrosine activity associated with Gq. However, despite the appearance of Src and G α q in the same immunocomplex, we cannot exclude the possibility that Src activation by G α q requires other protein. Our study also reveals that downstream of Src, activation of the ERK pathway by the CCK2R also requires the NPXXY motif.

The direct interaction of the Src SH3 domain with proline-rich motifs has been reported for the β 3 adrenergic and the purinergic P2Y2 receptors^[13,14]. Src family kinases also possess an SH2 domain that could directly bind to tyrosine-phosphorylated β 2 adrenergic receptor^[15]. We tested these two hypotheses in Src activation by the CCK2R. Indeed, the CCK2R has two proline-rich sequences which might interact with SH3 domains. In addition, it also has an ITIM-like motif within the C-terminus tail (LSYTTI) that is potentially phosphorylated on tyrosine residues, in turn leading to the recruitment of PLC γ through its SH2 domain^[16]. Mutation of the prolines into serines, as well as the replacement of the tyrosine 438 of the ITIM-like motif by a phenylalanine did not affect the activation of Src by the CCK2R in COS-7 cells (data not shown).

Transactivation of receptor tyrosine kinases is another mechanism by which G protein-coupled receptors activate Src family kinases. Recently, the CCK2R has been shown to transactivate the EGF receptor^[17-19]. However, this mechanism seems to be dependent on the cellular model. In the present study, we have observed that inhibition of the EGF receptor by AG1487 did not abolish CCK2R-mediated Src activation in AR42J and COS7 cells (data not shown).

Our study also shows that Src is activated upstream of the ERK pathway by the CCK2R in pancreatic tumor cells and contributes to the proliferative effects mediated by this receptor.

The development of cancer is thought to be dependent on the deregulation of normal signaling pathways involved in cell proliferation, thus conferring a growth advantage to cells. The role of Src and the ERK pathway in the regulation of cell growth is well documented. These signalling molecules have been implicated in the proliferative effects induced by tyrosine kinase receptors, cytokine receptors and G protein-coupled receptors. Activation of Src and ERK proteins has been observed in several human cancers and may contribute to the neoplastic phenotype. However, there is currently very little information regarding the role of the SRC/ERK pathway in the early stages of pancreatic carcinogenesis.

Here we report that the expression of the CCK2R in mouse pancreatic acinar tissue leads to strong activation of the Src tyrosine kinase and the ERK pathway. These transgenic mice display an increased growth of the pancreas

and preneoplastic lesions. They also develop pancreatic tumors with a ductal phenotype similar to what is observed in human pancreatic cancers. Deregulation of the Src/ERK pathway by the CCK2R in these transgenic mice might represent an early step that contributes to cell proliferation, formation of preneoplastic lesions and pancreatic tumor development. In addition, while several studies have reported the *in vitro* activation of Src and ERKs by gastrin^[10,11,20], this study is the first to demonstrate *in vivo* the up-regulation of the Src/ERK pathway by CCK2R.

In summary, our study describes the mechanism by which the CCK2R, a GPCR mainly coupled to Gq, activates Src. Our results show the involvement of the NPXXY motif within the receptor sequence in this activation, and suggest the putative role of Gq in this mechanism. Moreover, in pancreatic tumoral models we demonstrate *in vitro* and *in vivo* that the CCK2R activates the Src/ERK signaling pathway, a transduction cascade upregulated during tumorigenesis in human.

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BASIC RESEARCH

Effect of rapamycin on hepatic osteodystrophy in rats with portasystemic shunting

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were measured. In addition, the roles of IGF-1 and hypogonadism were investigated.

RESULTS: Portasystemic shunting caused low turnover osteoporosis that was RANKL independent. Bone resorbing cytokine levels, including IL-1, IL-6 and TNF α , were not increased in serum and TNF α and RANKL expression were not upregulated in PBMC. Portasystemic shunting increased the circulating CD8+ T-cell population. Rapamycin decreased the circulating CD8+ T-cell population, increased CD8+ CD25+ T-regulatory cell population and improved all parameters of bone turnover.

CONCLUSION: Osteoporosis caused by portasystemic shunting may be partially ameliorated by rapamycin in the rat model of hepatic osteodystrophy.

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Key words: Osteopenia; Liver disease; Portasystemic shunting; T-lymphocyte; Rapamycin

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Abstract

AIM: To study if T-cell activation related to portasystemic shunting causes osteoclast-mediated bone loss through RANKL-dependent pathways. We also investigated if T-cell inhibition using rapamycin would protect against bone loss in rats.

METHODS: Portasystemic shunting was performed in male Sprague-Dawley rats and rapamycin 0.1 mg/kg was administered for 15 wk by gavage. Rats received powdered chow and supplemental feeds to prevent the effects of malnutrition on bone composition. Weight gain and growth was restored after surgery in shunted animals. At termination, biochemical parameters of bone turnover and quantitative bone histology were assessed. Markers of T-cell activation, inflammatory cytokine production, and RANKL-dependent pathways

INTRODUCTION

Metabolic bone disease is a common complication of longstanding liver disease^[1,2]. Mechanisms underlying bone loss remain poorly understood and may involve imbalances in bone turnover and mineralization defects. We have shown that portasystemic shunting, a complication of advanced chronic liver disease, is a major pathogenic factor causing bone loss in rats^[3].

Excessive osteoclast activity, resulting in localized or generalized bone loss, occurs in various conditions associated with immune activation^[4,5]. Activated T-cells express receptor activator of nuclear factor- κ B ligand (RANKL) that bind to RANKL receptor on osteoclasts

activating osteoclastogenesis and bone loss^[4]. This pathway produces TNF α , IL-1, IL-6, IL-7, and M-CSF all of which have been implicated in bone loss^[6-10]. Blockage of RANKL by osteoprotegerin protects against bone loss^[4].

The profound impact of activated T-cells on bone has been established. Osteopenia develops in cta 4-/- mice where T-cells are spontaneously activated, whereas bone loss fails to occur in T-cell deficient nude mice even following ovariectomy^[11]. Further, RANKL expressing T-lymphocytes obtained from diseased rheumatoid arthritis joints transform healthy monocytes into osteoclast-like cells^[12]. We hypothesized that endotoxin-mediated T-cell activation related to portasystemic shunting may result in osteoclast-mediated bone loss through RANKL dependent pathways.

Employing biochemical parameters of bone turnover and quantitative bone histology, this study aimed to characterize the nature of the bone disease resulting from portasystemic shunting in rats. The role of T-cell activation and inflammatory cytokine production on RANKL-dependent pathways were specifically addressed.

MATERIALS AND METHODS

Animal Experimental design

Ten-week-old male Sprague-Dawley rats weighing 200-300 g were used in all experiments. The rats were housed individually in polypropylene cages at constant room temperature (22°C \pm 2°C), humidity (55%) and 12 h light-darkness cycles. Rats were fed powdered chow (Epol, Johannesburg, South Africa) and water was given *ad libitum*. Rats received a daily 50 mL supplement (Energy 44.52 kJ, protein 0.373 g, carbohydrates 1.456 g, fat 0.373 g). Food intake was measured daily in metabolic cages during four time periods: wk 3, 9, 12 and 15. Ethics committee approval was obtained and animals were treated according to ethical guidelines of the University of Pretoria.

Group I: $n = 12$ PSS. Laparotomy was performed, portal vein was ligated, transected and the distal limb anastomosed end-to-side to the IVC as previously described^[3,13].

Group II: $n = 12$ PSS + rapamycin. Portasystemic shunt was performed and rapamycin 0.1 mg/kg administered daily orally by gavage for 15 wk starting 1 wk after surgery. Two rats died during the study period.

Group III: $n = 12$ Sham control. Laparotomy was performed and the portal vein was clamped for 8 min.

Group IV: $n = 12$ Sham control + rapamycin. Following laparotomy controls received rapamycin 0.1 mg/kg, orally by gavage starting 1 wk following surgery and continued for 15 wk.

Analytical methods in sera and urine

Blood and urine samples were obtained at baseline and termination and frozen at -70°C. Urine was collected from rats individually housed in metabolic cages. Routine liver tests and testosterone were performed using a Beckman CX-9 and Access auto-analyzers respectively. 25-OH Vit D was determined using scintillation counting detection^[14]. Osteocalcin was measured using ELISA kit (Osteometer

BioTech, Herlev, Denmark). Cytokine levels were analyzed using an ELISA kit (Biotrac, Amersham, Buckinghamshire, United Kingdom). IGF-1 was measured using an ELISA kit (DRG Inc, Mountainside, USA). Urinary deoxypyridinoline was assessed using an enzyme-labeled immunoassay (Immunolite Pyrilinks-D, Los Angeles, USA).

Liver histology

Rat liver specimens were fixed in 40 g/L buffered formaldehyde and sectioned coronally in 3 μ m sections for immunoperoxidase staining utilizing antibodies to ED-1 (1:50 dilution; Serotec, Oxford, UK). Kupffer cells were counted by an experienced histopathologist in ten high power fields (Olympus BX 40, plan 40 x objective) demonstrating the most Kupffer cells. An average per high power field was then calculated.

Bone densitometry

Rats were anaesthetized and bone densitometry was performed using DEXA (DXA QDR 4500TM, Hologic INC, Waltham, USA). Measurement stability was controlled daily by scanning a phantom. Whole-body and high-resolution scans of the right femur were performed at baseline and 16 wk using software for small animals (Hologic, INC, Waltham, USA).

Histomorphometry

Rats received intramuscular injections of 25 mg/kg tetracycline 13 and 3 d prior to termination. At termination the left tibia was removed, stored in 70% ethanol at 4°C, fixed in modified Millonig solution for 24 h, embedded in methyl methacrylate, sectioned at 5 μ m and stained by modified Masson technique. Histomorphometric analyses were performed manually using a Merz-Schenk integrating eyepiece. Trabecular bone was analyzed excluding sections within two fields at $\times 250$ magnification from either the growth plate or the cortices. At least 120 fields per animal were counted. Double tetracycline-labeling was assessed on 10 μ m thick unstained sections cut from the proximal tibia by fluorescent microscopy using a Merz-Schenk eyepiece. Variables and units used are approved by the American Society for Bone and Mineral Research^[15].

Flow cytometry

Peripheral blood obtained at termination were incubated with the following combinations of monoclonal antibodies: fluorescein isothiocyanate/phycoerythrin labeled CD4+/CD25+; CD8+/CD25+ (Immunotech, Beckman Coulter, Inc. Fullerton, USA). Analysis was performed on a Coulter Epics flow cytometer. Lymphocytes were gated on forward and side scatter. The percentage of CD3+ T-cells in the gate was deduced from the percentages for CD4+ and CD8+ T-cells.

Quantitative RT-PCR

Total RNA was extracted from PBMC using the Qiagen RNeasy kit. Aliquots of 8.5 μ L were used in a RT-PCR reaction in a total volume of 20 μ L (Roche, First strand cDNA synthesis kit, Mannheim, Germany).

Complementary DNA PCR primers were designed from sequences from Genbank or TIGR (Inqaba Biotech, Pretoria, South Africa). *tnf α* (Sense: 5'-atggcccagaccctcac-3', Antisense: 5'-agccatagacggggcag-3'); *rankl* (Sense: 5'-tgga ggattttcaagctccgg-3'; Antisense: 5'-gccccaaagtacgtcgca-3') and *Gapdh* (Sense: 5'-ggccccctctggaaagct-3'; Antisense 5'-aggtggaggaatgggagt-3'). The reaction mixture consisted of cDNA (1 μ L), 10 pmol of each primer, 1 μ L LightCycler FastStart DNA Master SYBR Green 1 Mix (Roche, Mannheim, Germany), 500 ng BSA (Gibco, BRL, Gaithersburg, MD) and 3 mol/L MgCl₂ in a total volume of 10 μ L. FastStart polymerase was activated and cDNA denatured by a pre-incubation of 10 min at 95°C. The template was amplified for 40 cycles of denaturation at 95°C for 0 s, annealing at 60°C for 8 s and extension at 72°C for 12 s. Standard curves were generated from series diluted cDNAs and analyzed using the Light Cycler quantification software.

Analytical methods of bone calcium content

Right femurs were dried for 6 h at 60°C, then ashed for 8 h in a muffle furnace at 600°C. Bones were weighed, and the length and mid-shaft thickness measured. The femurs were dissolved in 3 mL of 6mol/L HCl and diluted 3000x with demineralized/de-ionized water. Calcium content was determined against a 4 point standard curve ($r^2 = 0.9999$), using a Perkin-Elmer 3030 atomic absorption spectrophotometer as previously described by our group^[3].

Determination of 4-hydroxyproline in left femurs

Dried femurs were dissolved in 10 mL 6 mol/L HCl, at 100°C for 24 h and centrifuged for 5 min at 4000 r/min at 4°C. n-Tetracosane (C₂₄H₅₀) in chloroform was added to the eluates as internal standard. The eluates were dried in nitrogen, the amino acids derivatized with N-methyl-N (*t*-butyldimethylsilyl) trifluoroacetamide and analyzed using gas chromatography as previously reported by our group^[16].

Statistical analysis

Data were analyzed using SigmaStat and SigmaPlot for Windows version 4.0. Data were checked for normality and equal variance; if passed, ANOVA was performed and where failed, analysis of variance on ranks (Kruskal-Wallis) was conducted. Results are presented as means \pm standard deviation. Analysis of covariance was performed to compare groups with respect to change in BMD from wk 1 to 16. Pair-wise comparisons were performed using Fisher's LSD and for between group comparison Wilcoxon rank sum test was performed. Results were considered significant at $P < 0.05$.

RESULTS

Body mass and food intake

Body mass decreased after surgery in the portosystemic shunted animals despite feeding with powdered and supplemental feeds. Weight gain was restored by wk 3 in the shunted animals and remained parallel to the control groups throughout the study (Figure 1), confirming growth in shunted animals. The rate of weight gain from

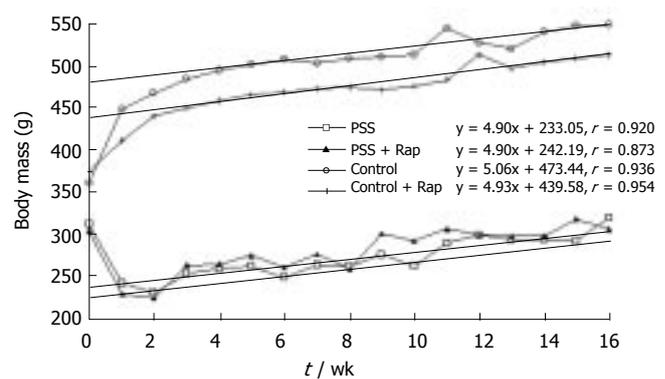


Figure 1 Body mass change per week. r = Pearson correlation coefficient.

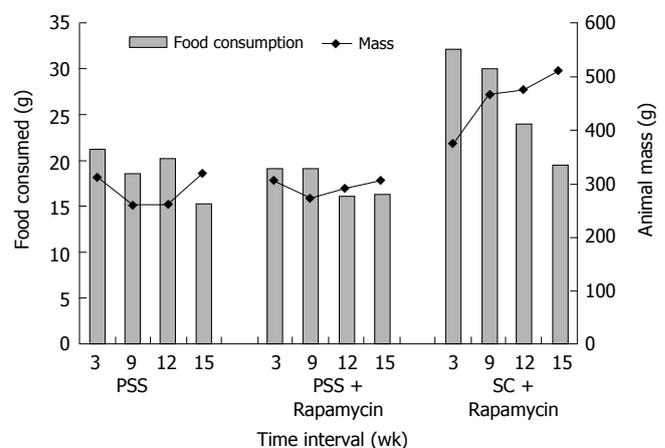


Figure 2 Food intake and body weight in PSS, PSS + rapamycin and SC + Rapamycin.

wk 2 as reflected in the Pearson correlation coefficient did not differ between the shunted and control animals (Figure 1). Powderized food intake remained lower in shunted animals during the first 12 wk of the study ($P < 0.01$). The supplemental feed was consumed by all animals daily. Food intake, corrected for body mass, showed no differences between the groups with total food intake (powderized + supplemental) being higher than the recommended intake for adult laboratory rats (15 g/d). Calcium and Vit D was further supplemented in the powdered chow. By wk 15 no difference in food consumption (powderized feed) was seen (Figure 2). Body mass was still significantly lower in shunted animals at termination (Figure 1).

Serum and urine biochemistries

No differences were seen at baseline. Transaminase levels were elevated in shunted groups and the control group receiving rapamycin. Serum total ALP was elevated in both shunted groups and confirmed to be of hepatic origin by electrophoresis. Serum albumin, testosterone and IGF-1 levels were significantly lower in both shunted groups. 25-OH vit D levels were not different between the groups at 16 wk (Table 1). Serum osteocalcin was significantly lower in the PSS group but not different in the PSS group receiving rapamycin compared to controls (Table 1). A trend towards higher u-DPD levels was observed in

Table 1 Serum and urine biochemistries at 16 wk

	ALP (nkat)	AST (nkat)	ALT (nkat)	Albumin (g/L)	Testosterone ng/L	IGF-I μg/L	25-OH vit D ng/L	Osteocalcin ng/L	u-deoxypyridinoline (μmol/mol creatinine)
Control	2161 ± 369	1182 ± 277	940 ± 187	16.75 ± 0.71	5.41 ± 2.34	879.37 ± 245.51	25.63 ± 4.04	142.91 ± 32.9	48.8 ± 7.9
Control + rapamycin	2762 ± 389 ^a	2022 ± 1152 ^a	1155 ± 122 ^a	15.78 ± 1.3	10.62 ± 9.1	616.02 ± 189.66	29.15 ± 8.00	147.43 ± 34.0	67.12 ± 13.6
PSS	7144 ± 2121 ^b	2210 ± 741 ^a	1538 ± 243 ^b	15.25 ± 0.75 ^a	3.55 ± 0.41 ^a	290.28 ± 170.90 ^a	23.89 ± 5.97	105.57 ± 38.3 ^a	61.99 ± 18.7
PSS + rapamycin	8481 ± 3380 ^b	3217 ± 1560 ^a	1752 ± 549 ^a	14.33 ± 1.22 ^a	3.45 ± 0.74 ^b	241.66 ± 80.58 ^a	22.15 ± 5.07	119.61 ± 43.5	93.79 ± 30.6 ^a

^a*P* < 0.05 vs Controls; ^b*P* < 0.001 vs Controls.

Table 2 Bone analysis at 16 wk

	Whole body BMD mg/cm ²	High resolution BMD mg/cm ²	Femur length (mm)	Mid-shaft thickness (mm)	Femur mass (g)/kg body mass	Ca ²⁺ mg/g bone	Hyp/g bone
Control (<i>n</i> = 12)	211.7 ± 9	379.7 ± 46	42.8 ± 1.1	4.7 ± 0.3	1.49 ± 0.1	260 ± 7	3576 ± 1980
Control + rapamycin (<i>n</i> = 12)	210.0 ± 8	377.2 ± 43	42.8 ± 1.3	4.7 ± 0.3	1.53 ± 0.1	258 ± 9	6104 ± 1600 ^a
PSS (<i>n</i> = 11)	201.9 ± 8 ^a	309.3 ± 63 ^a	36.6 ± 0.6 ^b	4.1 ± 0.3 ^a	1.80 ± 0.2 ^a	289 ± 5 ^b	2038 ± 878
PSS + Rapamycin (<i>n</i> = 10)	210.5 ± 17	334. ± 64	37.3 ± 1 ^b	3.8 ± 0.3 ^b	1.81 ± 1.37 ^b	297 ± 9 ^b	5489 ± 1250 ^a

^a*P* < 0.05 vs Controls; ^b*P* < 0.001 vs Controls.

Table 3 Quantitative histomorphometry

	Osteoid volume, OV/BV (%)	Relative osteoid volume, OV/TV (%)	Osteoid surface, OS/BS (%)	Osteoid thickness, O.Th (μm)	Osteoblast appositional rate, OAR (μm/d)	Mineralization lag time, MLT (d)	Eroded surfaces, ES/BS (%)	Osteoclastic surfaces, OcS/BS (%)
Control	1.26 ± 0.24	0.15 ± 0.04	8.94 ± 1.27	5.67 ± 0.65	1.03 ± 0.09	5.61 ± 1.08	5.63 ± 0.55	1.07 ± 0.20
Control + Rapamycin	1.34 ± 0.38	0.19 ± 0.06	10.44 ± 1.86	5.30 ± 0.93	1.05 ± 0.08	5.06 ± 1.25	6.35 ± 0.81	1.42 ± 0.24
PSS	3.69 ± 1.43	0.66 ± 0.32	17.48 ± 4.96	8.00 ± 1.32	0.85 ± 0.86	19.7 ± 5.07 ^a	3.62 ± 0.63 ^a	0.97 ± 0.28
PSS + Rapamycin	1.33 ± 0.19	0.15 ± 0.02	10.05 ± 1.53	5.72 ± 0.86	0.95 ± 0.11	11.02 ± 1.99	4.40 ± 0.62	0.94 ± 0.19

^a*P* < 0.05 vs Controls.

shunted animals compared to control animals. Significantly higher u-DPD levels were seen in control animals receiving rapamycin.

DEXA, bone mass and composition

Mean whole body and femoral BMD were comparable at baseline. At 16 wk whole body and high resolution femoral BMD were significantly lower in portasystemic animals compared to controls (Table 2). BMD in PSS receiving rapamycin did not differ from controls. The change in BMD from baseline to wk 16 using pair-wise comparisons showed that the increase in BMD in the shunted group (group I) was significantly lower compared to the control group (*P* = 0.006). The increase in BMD in the PSS receiving rapamycin was comparable to controls (*P* = 0.07). Shunted animals had lower body mass, femoral length and thickness (Table 2). Femur mass, expressed as a function of body mass, was increased in PSS. Femoral calcium content, expressed as a function of femoral mass (mg Ca²⁺/mg femur), was not decreased in shunted animals, excluding significant osteomalacia. Femoral hydroxyproline levels tended to be lower in the PSS group compared with controls (Table 2). Unexpectedly, shunted and control rats receiving rapamycin had significantly higher femoral

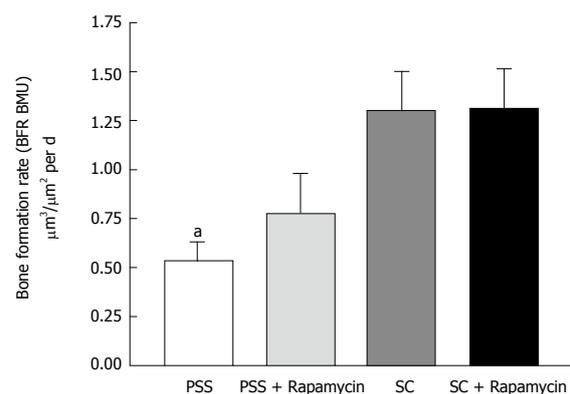


Figure 3 Effect of portasystemic shunting and rapamycin administration on dynamic bone formation in the proximal tibias of rats. BFRBMU = Bone formation rate at the level of the basic multi-cellular unit. (mean ± SE. ^a*P* < 0.05 vs SC).

hydroxyproline.

Quantitative Histomorphometry

Osteoid volumes and surfaces, and mean osteoid seam thickness, was higher in the PSS compared with control animals (Table 3). Bone formation was decreased in PSS rats (Figure 3). Mineralization of newly formed osteoid,

Table 4 Cytokine levels, T-lymphocyte subsets and Kupffer cell populations at 16 wk

	IL-1 (ng/L)	IL-6 (ng/L)	TNF α (ng/L)	%CD3+	%CD4+	%CD8+	Ratio CD4+ CD8+	%CD4+ expressing CD25	%CD8+ expressing CD25	Kupffer cells /10 high power fields
Control	22.28 \pm 4.39	0	13.6 \pm 2.73	74.05 \pm 5.49	43.15 \pm 3.93	30.9 \pm 7.05	1.4	7.96 \pm 2.99 ^d	10.62 \pm 2.64 ^f	46.26 \pm 7.95
Control + rapamycin	25.86 \pm 8.71	0	14.84 \pm 2.35	73.34 \pm 7.73	47.86 \pm 8.31	25.48 \pm 6.4	1.8	15.54 \pm 9.5 ^d	17.9 \pm 13.87 ^f	40.3 \pm 8.46
PSS	22.23 \pm 5.12	0.025 \pm 0.045	26.15 \pm 45.97	93.4 \pm 7.97 ^a	58.96 \pm 9.02	34.47 \pm 6.59 ^b	1.6	5.38 \pm 2.47 ^c	6.05 \pm 1.99 ^e	43.45 \pm 8.13
PSS + Rapamycin	26.12 \pm 6.21	0.011 \pm 0.033	15.5 \pm 11.83	73.19 \pm 5.66	56.78 \pm 5.60	18.39 \pm 5.73 ^b	3.1	11.38 \pm 6.96 ^c	19.10 \pm 14.58 ^e	44.95 \pm 9.16

%CD3 + T cells PSS vs controls ^b $P < 0.001$ vs Controls; %CD8+ T cells PSS vs PSS + Rapamycin ^d $P < 0.001$; %CD4 + CD25 + T cells PSS vs PSS + Rapamycin ^f $P < 0.001$; %CD4 + CD25 + T cells Control vs Control + Rapamycin ^b $P < 0.001$; %CD8 + CD25 + T cells PSS vs PSS + Rapamycin ^b $P < 0.001$; %CD8 + CD25 + T cells Control vs Control + Rapamycin ^k $P < 0.001$.

reflected in the mineralization lag time, was significantly delayed in PSS rats, accounting for the accumulation of osteoid in these animals (Table 3). Bone resorption over 16 wk, as reflected in the total eroded surfaces, was significantly decreased in PSS animals, although active osteoclastic resorption at the time of sacrifice was comparable among groups (Table 3). Bone turnover in shunted animals was increased by rapamycin as evidenced by increase bone formation and resorption. Osteoid accumulation and the mineralization defect induced by shunting were largely normalized by rapamycin (Table 3).

T-cells, cytokine levels and *tnf α* and *rankl* expression

PSS increased the percentage of circulating CD3+ lymphocytes by 1.3 fold ($P < 0.001$) above that for control rats (Table 4). Rapamycin treatment of PSS rats returned the CD3+ T-cell population to that of control rats with an altered ratio of CD4+ to CD8+ T-cells of 3:1. No alteration occurred in either the size of the CD3+ population or the normal ratio of CD4+ to CD8+ T-cells in rapamycin treated control rats (Table 4). PSS did not affect the normal CD4+ or CD8+ T-cells fractions expressing CD25. There was a significant increase ($P < 0.001$) in the percentage of CD4+ and CD8+ T-cells expressing CD25 with rapamycin treatment in both PSS and control rats (Table 4). Serum levels of IL-1, IL-6 and TNF α were similar in all groups (Table 4). RT-PCR showed that TNF α gene expression in shunted and control animals was comparable. RANKL-expression was significantly lower in PSS animals.

DISCUSSION

We previously investigated the contribution of parenchymal inflammation, portal hypertension and portasystemic shunting to the development of metabolic bone disease using rat models. Only portasystemic shunting caused significant bone loss^[3]. The present study aimed to delineate the immune responses associated with portasystemic shunting and how this may influence bone loss in the rat. Further, the profound effect of activated T-cells on bone turnover has been well established^[11,12]. We set out to study the effect of T-cell inhibition using rapamycin, not shown to have adverse effects on bone composition (in contrast to cyclosporin and tacrolimus), on biochemical and histological bone parameters in rats

following portasystemic shunting.

The present study confirmed the adverse effect of portasystemic shunting on skeletal integrity as evident by a significant decrease in whole-body and femoral BMD. Quantitative bone histology documented hyperostoidosis and an increased mineralization lag time in shunted animals, suggesting a degree of impaired mineralization. Histological evidence of frank osteomalacia was absent. Serum 25-OH vitamin D levels and femoral calcium content were comparable between shunted and control animals, and ALP iso-enzymes in shunted animals originated from liver not bone, supporting the histological evidence that the low BMD observed in shunted animals was mainly the result of osteoporosis.

Femur mass per 100 g body mass was higher in shunted rats compared to controls. Trabecular bone loss is known to occur earlier and more extensive compared to cortical bone loss due to the large surface area available for resorption. The apparent discrepancy between the rise in femur mass per 100 g of body mass in portasystemic shunted animals may be explained by lean body mass being lost earlier and more rapidly than cortical bone mass so that a lag in cortical bone loss gives rise to an apparent increase in bone mass relative to body mass. Low-turnover osteoporosis may occur due to protein-energy malnutrition^[17]. IGF-1 and testosterone levels were decreased in the shunted rats. Malnutrition^[18] and liver disease^[19] have been associated with decreased circulating levels of IGF-1. Low testosterone levels have been previously documented in portasystemic shunted rats^[20] with osteoporosis^[3]. IGF-1 has previously been implicated in bone loss in chronic liver disease^[21,22].

It is not possible to exclude that malnutrition contributed to bone loss in shunted rats. Malnutrition is associated with advanced liver disease although muscle wasting independent of malnutrition may be a feature of liver cirrhosis^[23]. Reduced spontaneous locomotor activity due to altered histaminergic neurotransmission can reduce food intake in shunted rats^[24]. We could, however, document that food intake in the shunted animals met the nutritional requirements calculated for their body weights during four time periods. However, despite restoring growth and weight gain (Figure 1), osteoporosis still developed in the portasystemic shunted rats. This improved feeding protocol may explain the differences observed in the present study compared to

our previous study^[3] In our previous study weight loss was observed throughout the study period and TNF α levels were increased and vitamin D levels decreased in shunted animals. In the current study TNF α and vitamin D levels did not differ from the control animals suggesting a stable model due to improved feeding.

An important observation in the study was that portasystemic shunted animals receiving rapamycin had increased bone formation, whole body and high resolution BMD, as well as increased osteocalcin levels in serum and hydroxyproline content in femurs, compared to shunted animals not receiving rapamycin. These positive changes on bone turnover variables occurred despite lower body mass, IGF-1 and testosterone levels compared to controls. This finding further suggests that malnutrition and decreased IGF-1 and low testosterone levels can only partially explain osteoporosis observed in shunting.

Pro-inflammatory cytokines like IL-1, IL-6, TNF α and RANKL are known to activate osteoclastogenesis and cause high-turnover osteoporosis^[9-11,25]. Serum levels of IL-1, IL-6 and TNF α , and *tnf- α* and *rankl* gene expression in PBMC, were not increased in shunted rats. Instead, osteocalcin, a marker of bone formation, was significantly decreased and quantitative histomorphometry showed impaired bone formation, suggesting low-turnover bone disease in shunted rats. Collectively, these observations suggest that the pro-inflammatory cytokines studied, are not responsible for bone loss in portasystemic shunting.

Rapamycin decreased the circulating CD8+ T-cell population in PSS rats. Lymphocyte proliferation inhibition by rapamycin is known to be dependent on the activation pathway of the proliferative signal with CD8+ T-cell proliferation being more affected than CD4+ T-cell proliferation^[26-28]. The effect of rapamycin on the CD8+ T-cell population in PSS rats indicate that these cells are more activated than CD4+ T-cells.

Expression of the CD25 marker in this study does not appear to reflect cellular activation. There was no difference between the control group and PSS group with respect to fraction of CD4+ or CD8+ T-cells expressing the CD25 marker, suggesting that the cells expressing CD25 belong to a population of regulatory T-cells (Treg cells). Treg cells in mice and humans are associated with a restriction of most immune responses^[29]. No documentation of such cell populations in rats has been made. Rapamycin increased the percentage of CD4+ and CD8+ T-cells expressing CD25 for both normal and shunted rats. This has been proposed to be a component of the immunosuppressive properties of rapamycin^[30]. Although not much is known of the function of the CD8+CD25+ T-cell population in rodents, this population in humans has similar immunoregulatory characteristics to the CD4+CD25+ T-cell population^[31,32]. Murine studies indicate an inability of CD8+CD25+ T-cells to induce osteoclast differentiation^[33]. In shunted rats where the fraction of these cells was increased by rapamycin, increased bone formation was observed. An increase in this population of CD8+ T-cells by rapamycin in normal rats also increased hydroxyproline levels in femurs.

The overall changes in the lymphocyte population seen with rapamycin treatment of shunted rats are associated

with an increase in bone formation, whole body and high resolution BMD as well as higher circulating levels of osteocalcin and femoral hydroxyproline content. Although IL-1, IL-6, TNF α and RANKL do not appear to be involved in portasystemic shunting associated osteopenia, other cytokines that are modulated by rapamycin may be implicated. IFN γ suppresses osteoclast activity while macrophage production of IL-12, a major stimulator of lymphocyte IFN γ production is upregulated by rapamycin. T-cell production of IFN γ via an IL-12 mechanism may be important in the bone protection observed in rapamycin treated animals^[34,35]. An alternative explanation whereby rapamycin may increase bone formation is through mTOR a member of the phosphoinositide 3-kinase related kinase (PIKK) family, which plays a critical role in transducing proliferative signals mediated through the PI3K/Akt signalling pathway^[36]. The mTOR gene is expressed in osteoblasts^[37,38] and rapamycin has been shown to up-regulate growth factors like BMP-4 and latent TGF- β binding protein in certain cancer cell lines^[39]. This may present an additional pathway by which bone formation is stimulated by rapamycin.

In conclusion, we hypothesized that portasystemic shunting would result in T-cell activation, cytokine mediated osteoclastogenesis and high-turnover osteoporosis. Instead, we documented low-turnover osteoporosis, which was partially ameliorated by rapamycin. A better understanding of the direct mechanisms of rapamycin on bone is required.

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Pancreatic regenerating protein (reg I) and reg I receptor mRNA are upregulated in rat pancreas after induction of acute pancreatitis

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significant increase in reg I receptor mRNA expression with pancreatitis. Immunohistochemistry localized this increase to the ductal cells, islets, and acinar cells.

CONCLUSION: Acute pancreatitis results in increased tissue reg I protein levels localized to the acinar and ductal cells, and a parallel threefold induction of reg I receptor in the ductal cells, islets, and acinar cells. These changes suggest that induction of reg I and its receptor may be important for recovery from acute pancreatitis.

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Key words: Acute pancreatitis; Reg, reg receptor; Taurocholate; Regeneration

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Abstract

AIM: Pancreatic regenerating protein (reg I) stimulates pancreatic regeneration after pancreatectomy and is mitogenic to ductal and β -cells. This suggests that reg I and its receptor may play a role in recovery after pancreatic injury. We hypothesized that reg I and its receptor are induced in acute pancreatitis.

METHODS: Acute pancreatitis was induced in male Wistar rats by retrograde injection of 3% sodium taurocholate into the pancreatic duct. Pancreata and serum were collected 12, 24, and 36 hours after injection and from normal controls (4 rats/group). Reg I receptor mRNA, serum reg I protein, and tissue reg I protein levels were determined by Northern analysis, enzyme-linked immunosorbent assay (ELISA), and Western analysis, respectively. Immunohistochemistry was used to localize changes in reg I and its receptor.

RESULTS: Serum amylase levels and histology confirmed necrotizing pancreatitis in taurocholate treated rats. There was no statistically significant change in serum reg I concentrations from controls. However, Western blot demonstrated increased tissue levels of reg I at 24 and 36 h. This increase was localized primarily to the acinar cells and the ductal cells by immunohistochemistry. Northern blot demonstrated a

INTRODUCTION

Since its isolation in 1988 by Terazono *et al*^[1], much work has been done to elucidate the function of the regeneration protein, reg I. Reg I protein appears to function as a growth factor in the pancreas, as evidenced by its structural similarity to calcium-dependant lectins^[2], as well as its mitogenic effect on beta and ductal pancreatic cell lines^[3] and its ability to reverse surgically induced diabetes^[4,5]. Reg I gene expression has also been found in other tissues including gastric cells^[6], and rats with water immersion stress induced gastric lesions demonstrate an increase in reg I expression in gastric enterochromaffin-like cells during healing^[7]. Reg I is therefore involved in the regeneration and growth of gastrointestinal tissue. Furthermore, acute pancreatitis induces reg I gene expression and protein production in the pancreas^[8,9]. Recently, Kobayashi *et al* isolated the gene for reg I receptor^[10], which is part of the exostosin family of genes. Transfection of the gene into the pancreatic β -cell line RINm5F cells resulted in increased mitogenesis after exposure to reg I protein.

Since reg I and its receptor are linked to cellular

mitogenesis and may affect repair of damaged pancreas, we examined the role of their expression in acute pancreatitis.

MATERIALS AND METHODS

Induction of acute necrotizing pancreatitis

Acute necrotizing pancreatitis was induced in male Wistar rats using a 30 g/L sodium taurocholate solution according to a protocol described by Aho *et al* with slight modification^[11]. The animals (4 rats/group) were anesthetized with a 1:1 mixture of ketamine (100 mg/mL) and xylazine (20 g/L) dosed at 0.1 mL per 100 g of body mass injected subcutaneously. The abdominal cavity was then entered through a midline incision. The duodenum and pancreas were identified, the common biliopancreatic duct ligated at the hilum of the liver, and a duodenostomy made approximately 1 cm distal to the opening of the biliopancreatic duct into the duodenum. A polyethylene catheter with an inner diameter of 0.58 mm and an outer diameter of 0.965 mm was used to cannulate the pancreatic duct through the duodenostomy. A 30 g/L sodium taurocholate solution was slowly injected at a dose of 1 mL/kg at a constant infusion rate of 0.5 mL/min. The catheter was left in place for 30 min and then removed. The midline incision was then closed in a single layer closure. Control animals consisted of sham operated rats (open laparotomy with immediate closure) and healthy rats.

Harvesting of pancreas and serum

A total of 12 rats were operated on for induction of pancreatitis. Four rats were sacrificed at each time point (12, 24, and 36 h). Four rats per group (unoperated and sham operated) were used for controls. The pancreas was harvested and blood collected by cardiac puncture from each rat. A small portion of the head of the pancreas was fixed in formalin and sent for staining with hematoxylin and eosin (HE) and immunohistochemical staining with reg I mAb^[12]. The remaining pancreas was divided in half. One portion was immediately snap-frozen in liquid nitrogen and used for RNA isolation (below), and the other for protein isolation. This was performed by homogenization in buffer containing 125 mmol/L mannitol, 40 mmol/L sucrose, 5 mmol/L ethylenediamine tetraacetic acid (EDTA), and 5 mmol/L piperazine ethane sulfuric acid-Tris (PIPES-Tris) (pH 6.7). The homogenate was centrifuged at 4100 g for 30 min and the supernatant collected and frozen at -20°C. The concentration of total protein in the pancreas homogenates was quantified using the BioRad Protein Assay solution according to the manufacturer's protocol. The blood was centrifuged at 1800 g for 15 min. The supernatant (serum) was collected and stored at -20°C.

Serum amylase activity was determined by enzymatic reaction. Formaldehyde-fixed pancreatic specimens from each rat were stained with HE.

Preparation of reg I cDNA probe

Reg I cDNA probe was prepared by PCR using a plasmid with a reg I insert as a template^[3]. The sequences of the

oligonucleotide primers are 5'ACGCGTCGACTCATGACTCGCAACAAATATTTTC3' and 5'GGCACTGCAGTCAGGCTTTGAACTTGCAGAC3'. The PCR was carried out using digoxigenin-labeled uridine triphosphate (DIG-labeled UTP) (Roche Diagnostics, Mannheim, Germany) under the following conditions: denaturing at 94°C for 30 s, annealing at 57°C for 30 s, and elongation at 72°C for 1 min; 30 cycles were used.

Preparation of Reg I receptor cDNA probe

A Reg I receptor cDNA was prepared using double digestion of pCIneo-Reg I receptor cDNA plasmid^[10] with *Hind*III and *Not*I. Electrophoresis of the digestion complex was performed on 8 g/L agarose gel, the receptor band cut from the gel, and the cDNA extracted from the gel using the QIAEX II agarose gel extraction protocol (Qiagen, Valencia, CA). The reg I receptor cDNA fragment was labeled with digoxigenin (DIG) using the DIG High Prime DNA Labeling and Detection Starter Kit II (Boehringer Mannheim, Germany) as per manufacturers' protocol.

Northern blot

RNA was extracted from rat pancreatic and intestinal tissue using Trizol reagent (Gibco, Life Technologies, Rockville, MD). RNA extraction was performed with 1-bromo-3-chloropropane. RNA concentrations were determined by absorbance measurements at 260 nm, and the integrity confirmed by polyacrylamide gel electrophoresis. Equal amounts (20 µg) of total RNA were then used for all Northern blots. The membrane was equilibrated in 10 × saline-sodium citrate (SSC) buffer for 5 min. The RNA samples were prepared as follows: 50 µL TE buffer (TE: 10 mmol/L Tris, 0.1 mmol/L EDTA, pH 8.0), 20 µL of 37 g/L formaldehyde, 30 µL of 20 × SSC, and 20 µg RNA. The samples were heated at 60°C for 15 min and immediately chilled on ice for 3 min. Two hundred microliters of 10 × SSC were then added to each tube. The samples were applied to the nylon membrane using a Northern slot blot apparatus. Each well was washed with 500 µL SSC. The membrane with RNA was UV cross-linked and placed in 50 mL of DIG pre-hybridization solution at 48°C for 6 h. Hybridization with the DIG-labeled reg I receptor cDNA probe was carried out at 58°C for 12 h. After washing and blocking, the membrane was incubated in a 1:10 000 dilution of anti-DIG-alkaline phosphatase conjugate antibody solution at room temperature for 30 min. CSPD solution (Boehringer Mannheim) was used for detection. Gels were photographed using Polaroid 667 film and digitized using an Epson 636 scanner. Band density analysis was performed using the public domain NIH Image program (available at <http://rsb.info.nih.gov/nih-image/>) to determine the quantity of nucleic acid product. To account for differences in the amounts of starting RNA between samples, the density of each reg band was normalized to that of the β-actin band for the same sample. Samples were run in duplicate and data are expressed as mean optical densitometric (OD) measurements ± SE.

Western blot on tissue homogenate to determine reg I levels

The tissue was homogenized in buffer containing 125

mmol/L mannitol, 40 mmol/L sucrose, 5 mmol/L EDTA, 5 mmol/L PIPES-Tris (pH 6.7), and 1 tablet of protease inhibitor cocktail per 50 mL of buffer. Total protein concentrations were determined using Bio-Rad protein assay. Electrophoresis was performed using equal amounts of total protein (10 μ g/well) in a 100 g/L sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE). The electrophoresis was carried out at 80 V for the first 15 min and then at 130 V for 45 min in SDS electrophoresis buffer. The protein was transferred to nitrocellulose membrane (Micron Separations, Inc., Westborough, MA) at 100 V for 1 h. The membrane was blocked overnight in 50 mL/L milk. A dilution of 1:5000 was used for the mouse anti-reg I mAb and incubated for 1 h with the membrane. After washing with T-TBS, the membrane was incubated for 45 min with a 1:2000 dilution of horse radish peroxidase-linked anti-mouse IgG. Detection was done using the ECL Western Blotting Detection System (Amersham Pharmacia, Piscataway, NJ). Human Reg 1 (hReg1) (10 μ g) was used as a positive antibody control.

ELISA on serum samples to determine reg I concentration

Each well of the microtiter plate was coated with a mixture of 50 μ L serum and 50 μ L of coating buffer (20 mmol/L Na_2CO_3 and 35 mmol/L NaHCO_3). Serial dilutions of human reg I were used as standards. The wells were coated overnight, followed by blocking with a 3% BSA solution for 4 h. The mouse reg I mAb was diluted 1:1000 and incubated for 1 h^[12,13]. A dilution of 1:1000 was used for the secondary antibody and incubated for 45 min. Detection was performed by adding 100 μ L of detection buffer (4.84 mL of 0.05 citric acid, 5.14 mL of 0.1 Na_2HPO_4 , 30 μ L of 300 mL/L peroxide, and 1 tablet of o-phenylenediamine) and measuring absorbance at 450 nm.

Development of anti-receptor antibodies

Two non-overlapping peptide sequences were identified that were contained in the extracellular domain of the Reg I receptor^[10] with predicted antigenicity and directly flanking specific amino acids thought to directly bind to Reg I (phage display experiment, unpublished data). PAELEKQLYSLPHWRTDC and RLLPEKDDA-GLPPPKATRGC were synthesized by solid phase synthesis (Biomolecules Midwest Inc., St. Louis, MO), purified by reverse phase high performance liquid chromatography (HPLC), and characterized by Mass Spectrometry. Peptides were coupled to KLH and used for immunization of rabbits. Rabbits were boosted at monthly intervals and bled 10-14 d after each boost. Antibody titer was determined in a microtiter plate ELISA using the conjugated peptide as the immobilized antigen. The immunoglobulin fraction was isolated using ImmunoPure (A/G) IgG purification system (Pierce, Rockford, IL) and the final antibody concentration was determined with the BCA Assay (Pierce).

Reg I receptor immunohistochemistry

Paraffin embedded sections were treated with Ficin (1:100 dilution, Sigma, St. Louis, MO) for 30 min at room temperature, washed, and were then treated with Avidin/Biotin blocking reagent (Vector Labs, Burlingame, CA) for

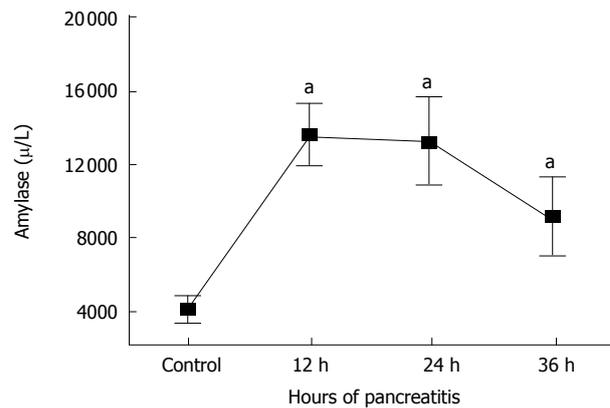


Figure 1 Serum amylase activity in pancreatitis rats (12, 24, 36 h) vs sham operated and normal controls. ^a $P < 0.05$.

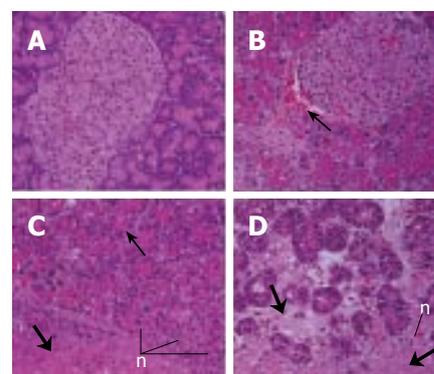


Figure 2 HE stain of normal rat pancreas (A) and after taurocholate injection at 12, 24, and 36 h (B, C, and D). Induction of pancreatitis resulted in hemorrhage (thin arrows), infiltration of neutrophils ("n"), and necrosis (thick arrows).

20 min. Slides were subsequently treated with a Protein Block Reagent (Dako, Carpinteria, CA) and incubated with primary antibodies overnight at 4°C. Goat anti-rabbit biotinylated IgG secondary antibody was applied for 1 h at room temperature (1:1500, NEN Life Science, Boston, MA). Endogenous peroxidase activity was quenched by treatment with 10 mL/L hydrogen peroxide/PBS and sections were then incubated with streptavidin-horseradish peroxidase (SA-HRP) (1:1000 dilution, Dako) for 30 min. For receptor experiments, tyramide amplification was applied for 3 min (NEN Life Science, Boston, MA). Slides were extensively rinsed and again incubated with SA-HRP (Dako, 1:1000) for 30 min at room temperature. Slides were developed in DAB (Sigma, St. Louis, MO) and counterstained with hematoxylin (Richard Allan, Kalamazoo, MI).

Statistical analysis

Data were expressed as means \pm SE. Student's *t* test was used for analysis. $P < 0.05$ was taken as significant.

RESULTS

The presence of acute necrotizing pancreatitis in taurocholate-treated rats was confirmed by serum amylase activity and histology (Figures 1 and 2). As demonstrated in Figure 1 serum amylase levels at 12, 24, and 36 h were elevated ($P < 0.05$) when compared to both sham operated and normal control rats. Figure 1 demonstrates a rapid increase

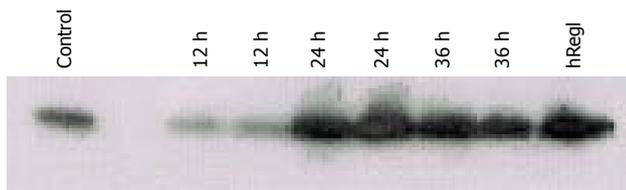


Figure 3 Slot blot analysis with anti-reg I antibody of total protein (10 μ g/well) from normal rat pancreas and rat pancreas after induction of acute pancreatitis. Reg I protein levels in tissue increased after 24 h of pancreatitis. Human Reg I (hRegI) is shown as a loading control as described in MATERIALS AND METHODS.

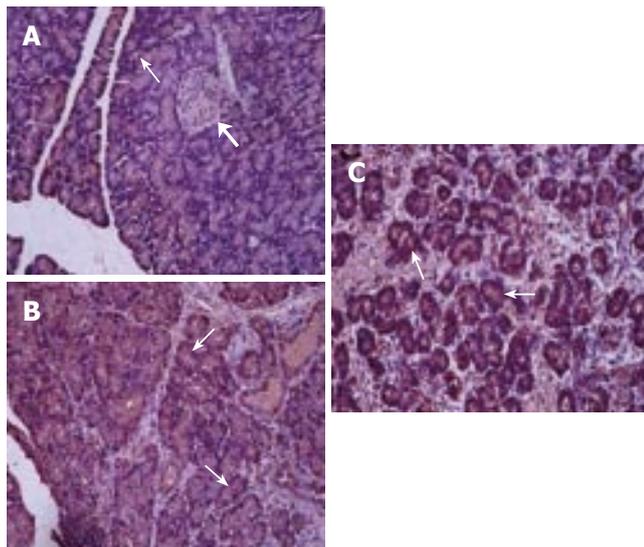


Figure 4 Immunohistochemical staining of normal rat pancreas with anti-reg antibody (A) and of rat pancreas after 24 and 36 h of pancreatitis (B, C). There is a marked increase in reg I levels in acinar cells of the pancreas after pancreatitis. Stained acinar cells are marked with thin arrows, compared with lack of staining in islet cells (thick arrow).

in amylase levels at 12 h with a gradual decline towards normal levels at 24 and 36 h. Figure 2 demonstrates histopathological worsening of pancreatitis in the pancreatic parenchyma after sodium taurocholate treatment as evidenced by hemorrhage (Figures 2 B, C) and necrosis (Figure 2D) when compared with control groups (Figure 2A).

Western blot analysis of pancreatic tissue showed a single band at 15-17 ku using a monoclonal antibody and demonstrated an increase in reg I protein in the 24 and 36 h pancreatitis groups compared to controls, although at 12 h, there was an initial decrease in reg I protein (Figure 3). Histologic examination showed that this appeared localized in the pancreatic acinar cells (Figure 4). Serum reg I protein levels were not significantly different in pancreatitis (0.43 ± 0.13 mg/L) when compared with sham-operated and normal controls (0.38 ± 0.15 mg/L) (data not shown).

Northern/slot blot analysis demonstrated a single band corresponding to the Reg I receptor mRNA and showed an increase in reg I receptor RNA expression in pancreatic tissue from the experimental pancreatitis groups compared to controls (Figure 5). Mean absorbance (A) \pm SE of reg I receptor signals on Northern/slot blot for control, 12, 24, and 36 h of pancreatitis were 24 ± 6 , 71 ± 15 , 66 ± 26 , and 75 ± 20 (all were significantly increased compared

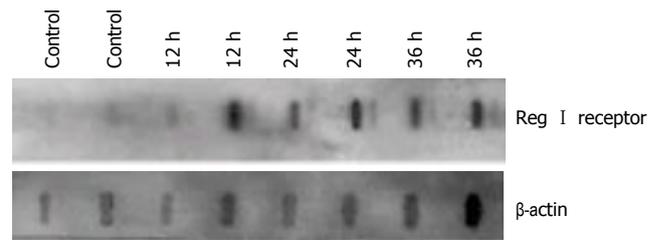


Figure 5 Slot blot of reg I receptor during pancreatitis showed an increase in reg I receptor mRNA expression after induction of pancreatitis. The density of each reg band was normalized to that of β -actin band for the same sample as described in MATERIALS AND METHODS.

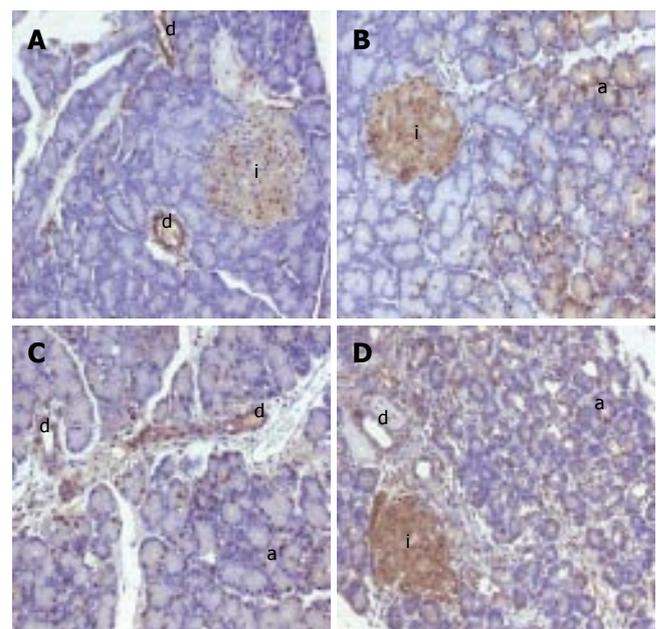


Figure 6 Immunohistochemical staining of pancreata from normal (A) and experimental pancreatitis (B-D) rats. There is an induction of reg I receptor expression in the acinar cells (a) and islets (i), and maintenance of reg I receptor expression in ductal cells (d) with pancreatitis.

to control, $P < 0.05$). Immunohistochemical staining with anti-reg I receptor antibody showed that although staining persisted in the ductal population, there was significant induction of receptor protein in both the islets and acinar cells (Figure 6).

DISCUSSION

Reg I is mitogenic to ductal and β -cells likely through the induction of the MAP kinase p38 pathway^[14]. This has been shown to be true in both cell culture and animal models of pancreatectomized rats. The data presented support the hypothesis that reg I is involved in regeneration after injury to the pancreas in the form of a sodium taurocholate-induced acute necrotizing pancreatitis. The pancreatic rats demonstrated an increase in reg I protein levels in pancreatic tissue after 24 h of pancreatitis. In the acutely inflamed pancreas it appeared that reg I protein was overproduced in the acinar cell population. This is consistent with the findings of others in both acute and chronic forms of pancreatitis^[15]. Although many genes are

differentially regulated in inflammatory states, microarray data by us and others have demonstrated increased Reg expression in experimental pancreatitis^[16,17]. Interestingly, serum reg I protein levels did not increase in parallel with acinar reg I RNA expression levels^[13]. This may be due to differences in cellular RNA turnover prior to stabilization of protein levels in serum, possibly through unidentified reg I binding proteins. Similar discord between protein and gene expression has been found in other systems^[18].

Baseline expression of reg I receptor is present in pancreatic ductal and beta cell lines, but not in acinar cell lines^[10,19]. These cells respond to reg I protein by proliferating, as measured by thymidine incorporation. Cells transfected with the receptor respond to reg I in a similar fashion^[10]. Acinar cells, which typically do not grow in response to reg I, do not express the receptor. It is therefore likely that the expression of the receptor is linked to the activity of cellular mitogenesis, and may be important in the proliferative response of pancreatic regeneration.

We found that pancreatitis significantly induced the expression of reg I receptor RNA in pancreatic tissue compared with baseline level. Immunohistochemical analysis with a polyclonal antibody to the reg I receptor localized the induced protein to the acinar cells and islets, and was only mildly present in ductal cells. This pattern of induction was in sharp contrast to the increase in reg I which was initially induced primarily in the acinar cells. While ductal cells typically proliferate after pancreatitis^[20], the additional induction of the protein in islets and acinar cells likely means that they are becoming responsive to the reg I protein which is being produced and secreted by the acinar cells.

Our present data suggest that increased reg I protein and receptor are important in the response of the pancreas to injury. Specifically, reg I may exert its mitogenic effect on all the cell populations after injury. It likely works in a paracrine fashion, since it is produced and secreted from acinar cells. The induction of the receptor after injury in the acinar cells and islets and its maintenance of expression in the ductal cells may allow the protein to enhance its regenerative effect.

Another new protein induced in pancreatitis is reg III, or pancreatitis associated protein (PAP). Seventy percent homologous to reg I, the pancreatic RNA and protein levels are increased even more dramatically in pancreatitis, and PAP protein is also increased in serum^[21,22]. We showed that the bovine form of reg III is mitogenic to pancreatic β - and ductal cells as well^[5]. It is possible that reg III protein interacts with the reg I receptor which may provide an important pathway for regeneration after injury.

Future experiments need to determine whether blocking the receptor will block the regenerative effect of reg I, and whether other members of the reg family, in particular reg III, can stimulate the receptor as well.

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Ultrasonographic study of mechanosensory properties in human esophagus during mechanical distension

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Abstract

AIM: To study the esophageal geometry and mechanosensation using endoscopic ultrasonography during volume-controlled ramp distensions in the distal esophagus.

METHODS: Twelve healthy volunteers underwent distension of a bag. During distension up to moderate pain the sensory intensity was assessed on a visual analogue scale (VAS). The esophageal deformation in terms of multidimensional stretch ratios and strains was calculated at different volumes and VAS levels. Distensions were done before and during administration of the anticholinergic drug butylscopolamine.

RESULTS: The stimulus-response (volume-VAS) curve did not differ without or with the administration of butylscopolamine. Analysis of stretch ratios demonstrated tensile stretch in circumferential direction, compression in radial direction and a small tensile stretch in longitudinal direction. A strain gradient existed throughout the esophageal wall with the largest circumferential deformation at the mucosal surface. The sensation intensity increased exponentially as function of the strains.

CONCLUSION: The method provides information of esophageal deformation gradients that correlate to the sensation intensity. Hence, it can be used to study mechanosensation in the human esophagus. Further studies are needed to determine the exact deformation stimulus for the esophageal mechanoreceptors.

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Key words: Deformation analysis; Esophagus; Experimental pain; Ultrasonography

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INTRODUCTION

Acute and chronic pain in the upper gastrointestinal tract is a severe and frequent health care problem in terms of human suffering and enormous economic implications for the health care system^[1]. Thorough understandings of visceral pain mechanisms are necessary for developing diagnostic tools and optimal treatments. However, pain and related symptoms are difficult to study due to the heterogeneity of patients and confounders related to clinical pain^[2].

Human experimental pain models can be used as tools for better characterization and understanding of pain mechanisms. In recent years studies have been focused on mechanical distension^[3-5]. However, many experimental pain studies of hollow visceral organs have reached erroneous conclusions since the pressure, volume and wall tension are proxies of the deformation in the vicinity of mechanoreceptors and therefore only show indirect degree of association with the evoked responses. It has been demonstrated in several parts of the digestive tract including the esophagus that deformation (circumferential strain) is more closely associated with the distension-induced sensory responses than the distension pressure, volume or wall tension^[6-8]. Calculation of the strain, however, demands advanced methods. Recently, endoscopic ultrasonography has been used to obtain information about the geometry of the esophagus including wall and layer thicknesses for the purpose of computing wall tension, stress and strain during swallowing^[9-12]. However, none of these studies has carefully evaluated the sensory properties during esophageal distension.

In the present study, endoscopic ultrasonography was combined with controlled esophageal distension and sensory assessment to obtain information about the mecha-

nosensation. Since deformation is considered the most important parameter for mechanosensation^[6-8], we measured the needed geometric variables and computed multidimensional stretch ratios and strains in order to obtain new information about the relationship between stretch and evoked pain.

MATERIALS AND METHODS

Subjects

Twelve healthy subjects, 6 males and 6 females, median age 38 years (range 22-67 years) recruited from the hospital and university staff, volunteered to participate in the study. They did not suffer from previous or current gastrointestinal disease and had no gastrointestinal complaints. The volunteers did not take any drugs and abuse alcohol. The regional ethics committee approved the study protocol and the experiments were conducted in accordance with the revised Declaration of Helsinki. All volunteers were asked to give a written informed consent to participate in the experiment.

Probe design

The probe was constructed from a thin flexible tube and equipped at the end with a highly compliant latex balloon. The tube was connected to an infusion pump (Type 111, Ole Dich Instrument makers, Hvidovre, Denmark). Saline heated to body temperature was infused into the balloon at the rate of 25 mL per min. The 6-cm long balloon was made of natural rubber with a thickness of 0.2 mm. In the deflated state the largest transversal diameter of the balloon was 10 mm. The shape of the balloon became almost spherical when inflated. An ultrasonographic miniprobe was advanced through the probe lumen and the transducer was placed inside the balloon. The ultrasound system used a rotating 20-MHz transducer (model UM-3R; Olympus, Japan). The ultrasound transducer produced a real time 360-degree cross-sectional image of the esophagus.

Protocol

The subjects were fasted at least for 6 h. Studies were performed with the subjects in half supine position. The probe was lubricated and inserted through the nostrils. The tip of the probe was first advanced into the stomach. The lower esophageal sphincter (LES) was identified by pull-through manometry as a zone of resting pressure that decreases with swallowing. The balloon was placed 8 cm proximal to LES and the probe was fixed to the nose to prevent migration of the assembly during the study.

Distensions were done to precondition the tissue and served as learning sessions to facilitate the sensory rating. Since preconditioning is necessary to obtain reproducible results^[4], three distensions using a constant infusion rate of 25 mL/min were done until the pain threshold was reached (5 on the visual analogue scale (VAS)). After the preconditioning distensions, two more distensions to 7 on the VAS (moderate pain level) were done. Then a bolus of 20 mg butylscopolamine was given intravenously to decrease distension-induced secondary smooth muscle contractions. The balloon was again inflated twice using

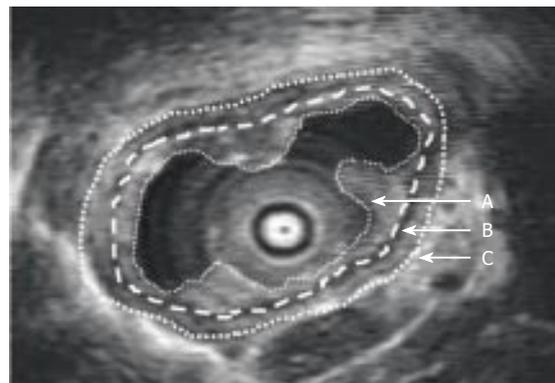


Figure 1 Transversal ultrasonographic image of the non-distended esophagus. The stippled lines indicate manual tracings of the mucosal inner surface (A) and the inner (B) and outer (C) lining of the combined muscle layers.

an infusion rate of 25 mL/min until 7 on the VAS was reached. The balloon was emptied for 2 min between the distensions. None of the volunteers experienced significant side effects due to the anti-cholinergic action of the drug. The total investigation time never exceeded 1.5 h.

Sensory assessment

The sensory intensity was assessed continuously during the mechanical distension by using an electronic VAS-meter (GMC-Medical, Hornslet, Denmark). The VAS data were amplified and analog-to-digital converted at a sampling rate of 10 Hz using the Openlab data acquisition system (GMC-Medical, Hornslet, Denmark). The digitized data were stored on a PC for later analysis.

Before commencement of the visceral mechanical stimulation, the subjects were trained in using the VAS. The sensations were produced by applying deep pressure in muscles of the right forearm. The intensity of non-painful stimuli was scored up to 5 on the VAS. Key anchor words to describe the sensations were: 1 = vague perception of mild sensation; 2 = definite perception of mild sensation; 3 = vague perception of moderate sensation; 4 = definite perception of moderate sensation; 5 = pain threshold. For the painful sensations the patients used the scale from 5-7 anchored at 6 = slight pain and 7 = moderate pain^[2].

Data analysis

Ultrasonographic images of the esophagus were recorded in real time during the studies using a super-VHS videotape recorder. The videotape recordings were captured in video streaming 120 + MB/s at a frame rate of 25 s⁻¹ and a resolution 756 × 576 by a high performance video grabber (I-Color, Image House A/S Copenhagen DK). All image measurements and calculations were performed off-line using an image analysis software package (Scion Image Beta 4.02 Win, Maryland). We selected one reference image at each pain intensity level from the distensions after preconditioning the tissue. The images were selected at points where contractions were not present. The mucosa and muscle layers were outlined manually (Figure 1). Once outlined, the software program automatically calculated the circumference and the area of the layers. The layer

thickness was computed from the circumferential length and area.

The steady state pressure, diameters, CSA and wall thickness values at each volume distension step were used for the computation of stretch ratios and strains.

The circumferential Cauchy strain ϵ was computed as the fractional change in circumference^[3].

$$e = \frac{C - C_0}{C_0} \quad (1)$$

where C is the circumference obtained at the serosal or mucosal surfaces or between layers at various degrees of distension, C_0 is the reference circumference obtained at unloaded conditions immediately prior to a distension. The strain in radial direction could be computed from the measured wall thickness h as

$$e = \frac{h - h_0}{h_0} \quad (2)$$

where h_0 is the reference thickness obtained at unloaded conditions immediately prior to a distension. In a similar way the strain could be computed for the individual wall layers.

In a mechanical analysis it may also be convenient to use the stretch ratio as a measure of deformation. The stretch ratio λ was determined for both the circumferential (θ), radial (r) and longitudinal (z) direction. The stretch ratio is defined as the length at loaded conditions (L) divided by a reference length at unloaded conditions (L_0).

$$\lambda = \frac{L}{L_0} \quad (3)$$

Thus the stretch ratio $\lambda = \epsilon + 1$. For circumferential and radial direction the stretch ratios could be determined from the experiments using the same principle as for the strain calculation. The stretch ratio in longitudinal direction λ_z was determined from the two other stretch ratios by using the law of mass conservation. Hence, $\lambda_z = 1/\lambda_\theta\lambda_r$ ^[3,4,13].

Statistical analysis

The results were expressed as mean \pm SE. At various volumes and VAS levels 1-7, the corresponding stretch ratios before and during administration of butylscopolamine were determined. Two-way-ANOVA was used for statistics. $P < 0.05$ was considered statistically significant.

RESULTS

Ultrasonographic and VAS data were obtained in all subjects. The distensions were done until moderate pain (VAS = 7) was experienced except in one subject in whom the distension was done only until the pain threshold (VAS = 5) was reached. Three subjects repeatedly got involuntary long lasting contractions in the esophagus after the pain threshold was reached. Butylscopolamine greatly reduced these contractions. One subject reported autonomic side-effects during the distensions manifested as tachycardia, nausea and sweating.

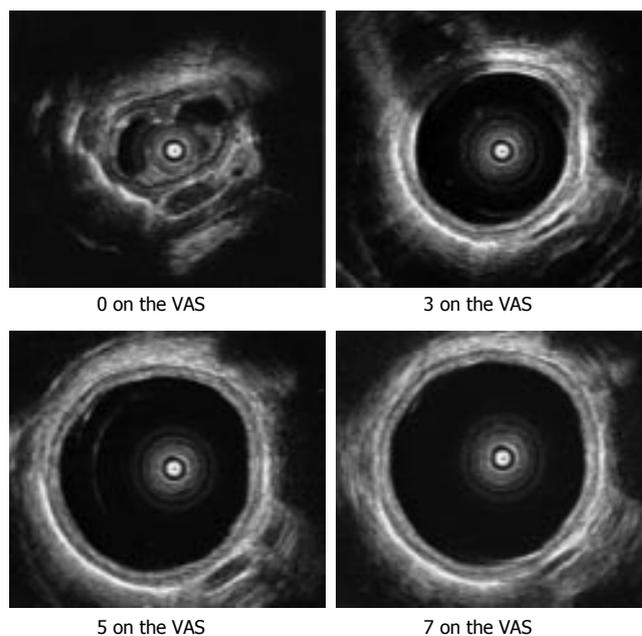


Figure 2 Transversal ultrasonographic images of the esophagus at different sensory perception levels. The esophagus is fairly circular at 3 on the visual analogue scale. The numbers refer to the sensory intensity at a visual analog scale with 5 as the pain threshold.

The preconditioning stimuli resulted in reproducible mechanical distensions and sensory-motor responses. Figure 2 shows the representative ultrasound images obtained at different VAS levels. The mean thickness of the mucosa and muscle layer in the resting state was 1.4 ± 0.02 mm and 2.4 ± 0.03 mm respectively. The esophageal folds almost disappeared at VAS level of 3 or higher where the geometry could be approximated as circular. The interface between the inner circular and outer longitudinal muscular layer could be visualized as a thin echogenic layer only at low volumes in the balloon. This layer was not consistently visible in the subjects at VAS levels of 2 or higher (Figure 2) and therefore could not be used in the strain analysis.

We found no difference in the volume-VAS data before and during butylscopolamine infusion (Figure 3-top, $F = 2.50$, $P = 0.12$). Consequently we used the data obtained during butylscopolamine infusion for the further analysis since contractions to some degree would bias the readings. Thus, the increased VAS primarily would be due to stretch of the wall rather than the active stress generated by the muscle tissue. Figure 3 also shows the circumferential, radial and longitudinal stretch ratios as function of volume and sensory intensity on the VAS for the mucosa and main muscle layers. In both layers, the circumferential deformation was tensile, whereas the radial deformation was compressive due to the decrease in layer thickness during the distensions. The computed longitudinal stretch ratio was tensile but less than the circumferential stretch ratio. Furthermore, the longitudinal stretch ratio did not increase further after 1 was reported on the VAS or a volume of 10 mL was reached. At the pain threshold (VAS 5) the mean circumferential, radial and longitudinal stretch ratios in the muscle layer were 1.7 ± 0.1 , 0.5 ± 0.04 and 1.2 ± 0.07 , respectively.

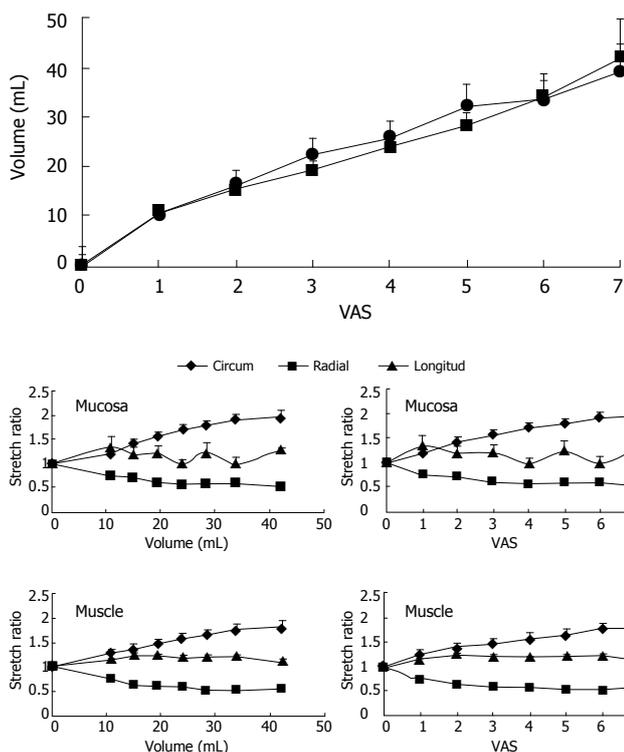


Figure 3 Illustration of the relation between volume and sensory intensity on the visual analogue scale (VAS) with 5 as the pain threshold (top graph). Since there was no difference in the curves obtained (triangles) before and during (squares) infusion of butylscopolamine, data obtained during butylscopolamine are given in this paper. The four figures show the circumferential, radial and longitudinal stretch ratios as function of volume and VAS in the mucosa and muscle layers (the circumferential data are from the mucosal surface and the outer muscle surface).

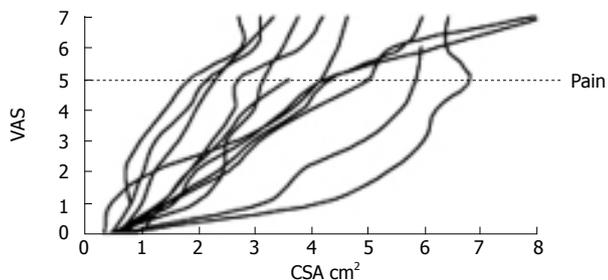


Figure 4 Sensory intensity on the visual analogue scale (VAS) with 5 as the pain threshold as function of the luminal cross-sectional area (CSA). A large variation was found in volunteers, which speaks in favor of using non-dimensional measures as the stretch ratio.

The sensory response showed a large variation between the volunteers. Figure 4 shows the sensation as function of luminal cross-sectional area (CSA). The rather large inter-individual variation speaks in favor of using non-dimensional measures such as stretch ratios rather than the volume or CSA. Figure 5 shows the circumferential stretch level at the mucosa and the inner and outer muscle layers. The circumferential deformation at the mucosal surface was larger than that at the outer muscle layer at high sensory levels. However, at low VAS levels the mucosal deformation was small, which was due to unfolding rather than stretch of the mucosa initially during the distensions. A strain gradient existed through the esophageal wall with

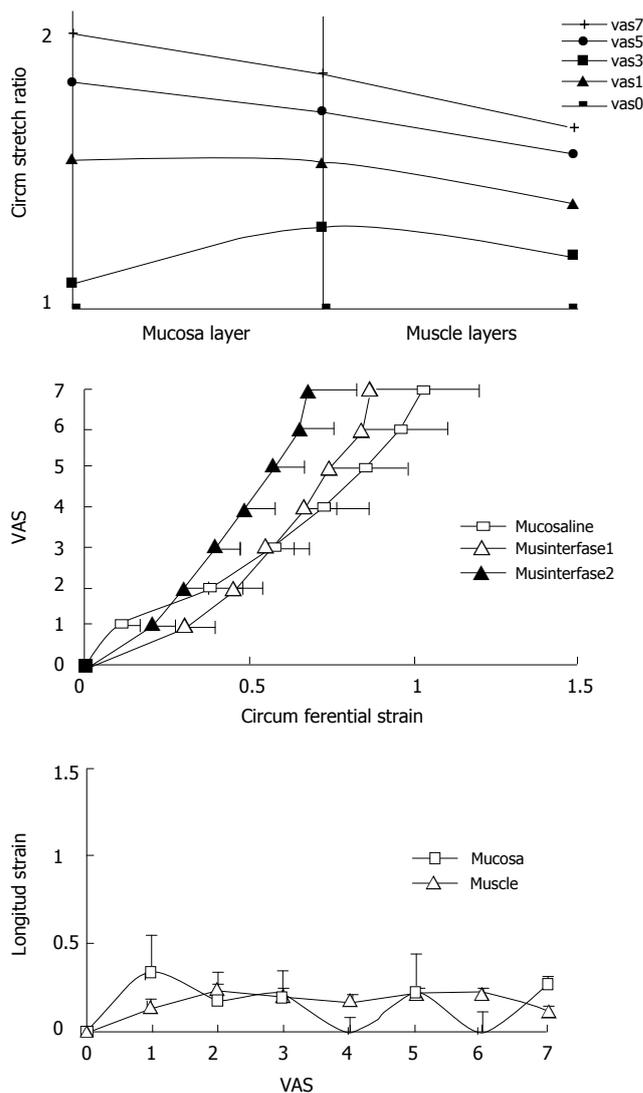


Figure 5 The top graph shows the circumferential stretch ratio at the mucosal surface (left), the interface between the submucosa and circumferential muscle layer (middle) and the serosal surface (right). The middle graph shows the same data in terms of the circumferential strain and sensory intensity on the visual analogue scale (VAS). There was a strain gradient throughout the esophageal wall with the largest deformation in the mucosa. The bottom graph shows the longitudinal strain as function of the sensory level. No increase in strain was seen during sensory intensity was increased. For convenience the data from all VAS are not shown.

the largest deformation in the mucosa and the sensation increased exponentially as function of the strain in both the mucosa and muscle layers (Figure 5). Longitudinal strain during distension seemed not to cause the sensation as the longitudinal strain did not increase further after VAS 1 (Figure 5 bottom). To evaluate further whether radial or circumferential strains were most important, we plotted the dispersion (SE/mean) of the radial and circumferential stretch ratio data from Figure 3. The circumferential stretch ratios in the muscle and mucosa layers showed the smallest dispersion (Figure 6).

DISCUSSION

The function of the esophagus is mechanical to a large extent. Swallowed contents are propelled through the esoph-

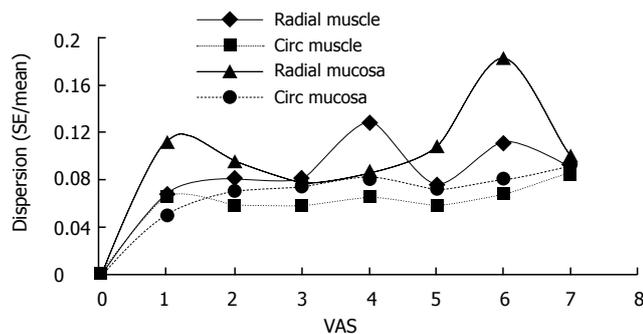


Figure 6 Dispersion (SE/mean) of the circumferential and radial strains in the mucosa and muscle. The dispersion was the lowest in circumferential direction.

agus into the stomach where the digestion starts. Methods traditionally used for clinical or basic investigations of the esophagus are endoscopy, manometry, pH-metry and radiographic examinations. Although these methods provide important data on esophageal motor function, little attention has been paid to the biomechanical and mechanosensory properties of the wall and determination of the wall deformation. During recent years ultrasonography has gained interest in studying gastrointestinal (GI) physiology and in clinical evaluation of the esophagus. In the current study, the stretch was tensile in circumferential direction, compressive in radial direction and tensile to a small degree in the longitudinal direction. A strain gradient existed throughout the esophageal wall with the largest circumferential deformation at the mucosal surface. The sensation intensity increased exponentially as function of the strains and the esophageal deformation gradients correlated to the sensation intensity.

Endoscopic ultrasonography is a non-invasive and safe method applicable in study of the esophagus. Two-dimensional B-mode ultrasonography has been utilized to assess the correlation between ultrasonographic images and histology^[13] and to determine biomechanical properties of the esophagus *in vitro*^[14]. Furthermore, Nicosia *et al*^[10] have used endoscopic ultrasonography in humans to study esophageal longitudinal muscle shortening and Miller *et al*^[11] have studied the correlation between wall changes and manometry during esophageal motility. Moreover, Pehlivanov *et al*^[9] showed that muscle geometry is changed during contractions in volunteers and patients with esophageal spasms. None of these studies has paid attention to the sensation provoked by balloon distension. Thus, despite these developments and the fact that the mechanical wall properties are important for normal function of the esophagus, the mechanosensory properties of the esophagus *in vivo* are still largely unknown. In this study we therefore intended to gain a better understanding of esophageal deformation and mechanosensory function.

Methodological aspects

In distensible biological tubes, the circumferential wall strain is of interest because the tensile stress is the largest in that direction during distension^[3,4,15]. The reasons for studying the stresses and strains in intact hollow organs are as follows: wall stress and strain data give valuable infor-

mation on the elastic properties^[4], mechanoreceptors do not respond directly to changes in pressure or volume but rather to changes in strain^[2], it is important to differentiate active properties such as phasic contractions and smooth muscle tone from passive tissue properties in pharmacological and biomechanical studies^[2,16,17], and intact organs maintain normal geometry in contrast to the tissue strips often used for length-tension measurements in physiological and pharmacological studies^[15,18].

In this study, we aimed to develop a new method for investigation of the mechanosensory properties in the esophagus, mainly in the state where contractions did not confound the analysis. The method is based on distension of a bag placed in the lumen with concomitant measurement of the distension pressure and the antral geometry in a selected cross-sectional plane by endoscopic real-time B-mode ultrasound. The ramp protocol ensured that we studied the elastic properties rather than viscoelastic properties^[4,15]. This ease the interpretation of the data since time-dependent confounders are significantly eliminated^[4]. The administration of the antimuscarinic drug butylscopolamine allowed us to investigate the active and passive tissue behavior. Since the volume-VAS curve did not differ between the distensions before and during butylscopolamine administration, we only analyzed the distensions after administration of butylscopolamine. This gives data that are easier to interpret as there is no significant influence of esophageal contractions. Butylscopolamine may not abolish all contractions. Therefore we analyzed the data between contractions if they were present. Since stretch as well as contractions may elicit pain, we primarily looked into the stretch-dependent pain mechanisms in this study. It has been found in other tissues as in the cardiovascular system that the baroreceptors depend on strains in the vicinity of the receptors^[15]. We have previously shown that strain is the most important biomechanical parameter for sensory responses in the gastrointestinal tract including the esophagus^[6-8]. Thus, in this study we were only concerned with the deformation as a stimulus for the mechanoreceptors.

The ultrasonographic technique makes the derivation of strains in multidimensional directions possible from the geometric data or due to use of the mass conservation principle. The strain calculations are based on direct measurements rather than on geometric assumptions such as a circular cross-section and the thickness of the layers measured in our study, which are consistent with the data presented by others^[10]. The fact that the lumen is not circular at low volumes makes it difficult to compute tension and stress based on Laplace's law^[3,4,15], but the strain analysis does not depend on the geometry as long as the surfaces and interfaces between layers can be clearly defined. In most cases we did not have any problems in identifying the tissue layers. Thus the error due to these measurements is considered small. However, the ultrasound images only show the primary convolution (folds) but not secondary and tertiary convolutions of the mucosa. If higher-order convolutions were present, this could result in a minor underestimation of the calculated stretch in the mucosa and a larger inter-individual variation. Unfortunately, we could

not clearly outline the interface between the two muscle layers at VAS levels above 3. Hence, we omitted presenting data on this interface.

Biomechanical and mechanosensory aspects

In the present study, the deformation was the largest in the circumferential direction, despite the presence of mucosal folds the circumferential deformation decreased through the wall with the largest values at the mucosal surface, and the sensation increased exponentially as function of the circumferential strain.

Gastrointestinal symptoms are often associated with disturbances in motility and sensory function. Several studies have attempted to investigate these properties by means of balloon distension^[19-21]. Unfortunately, the primary mechanism for symptoms elicited by GI distension remains unclear. It is well known that distension of the gastrointestinal tract elicits reflex-mediated inhibition and stimulation of motility via intrinsic or extrinsic neural circuits and induces visceral perceptions such as pain^[21]. Previous studies demonstrated that mechanoreceptors located in the intestinal wall play an important role in the stimulus-response function^[22-26]. It is, however, a common mistake to believe that mechanoreceptors are sensitive to variation in pressure, volume or tension. A large variation in the perception has been found in various studies and species^[27-29], suggesting that pressure is not the direct stimulus. The dogma that the mechanoreceptors are tension-sensitive receptors lying in-series or in-parallel with the muscle cells has also been prevalent for many years^[30]. This concept is borrowed from striated muscle physiologists and should yet be regarded as a working hypothesis, since no clear evidence supports it in GI studies. It is basically a uniaxial model that does not account for more complex biomechanical properties such as distribution of the deformation field and that different receptor populations may exist. There seems to be no evidence that the receptor is dependent of tension rather than strain. Instead, the receptors are stimulated by deformations acting on the intestinal wall due to changes in the transmural pressure^[31]. Thus, the mechanical distension stimulus and the biomechanical tissue properties must be taken into account in studies of the sensory-motor function in the esophagus.

It is well known that the passive elastic behavior of biological tissues is exponential^[3,4,15]. This mechanical feature protects the tissue against over-distension and damage at high luminal pressure loads while distending easily and facilitating flow in the physiological pressure range. In arteries, it has been demonstrated that collagen bears circumferential loads at high stress levels^[32,33]. As gastrointestinal tissue is rich in collagen^[34], it is likely that collagen is a major determinant of the passive mechanical properties. Though we did not evaluate the elastic properties in terms of stress-strain relations, the fact that the strain-VAS curve is exponential indicates that the exponential properties play a role in protecting tissue against high stress.

Our results may shed some light on the discussion regarding the visceral sensation receptors in human beings. The data suggest that at low loads the deformation occurs mainly in the muscle layer, whereas at higher loads,

the largest deformation occurs at the mucosal surface. Furthermore, the lowest dispersion has been found for the circumferential stretch ratio in the muscle layer. Though this analysis may be biased by measurement of errors for the two directions, the analysis may reveal that the most important mechanoreceptors are located in the muscle layer where they sense circumferential deformation. This view is also supported by other data showing that the circumferential deformation in the mucosa is much smaller than in the muscle layer at low loads, where the mucosa merely unfolds rather than stretches. However, the differences are small because the deformations in various directions depend on each other (due to the mass conservation principle) and require further analysis.

Conclusions and future clinical applications

We have developed a new method to study the strain-dependency of esophageal mechanoreceptors. The data point in the direction of a strain receptor sensitive to circumferential deformation in the muscle layer. The new test can be used to test drugs on esophageal mechanosensory function, and improve our understanding of the pathophysiology underlying the symptom induction in patients with pain and other symptoms relating to the esophagus.

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CLINICAL RESEARCH

Computed tomographic differentiation between alcoholic and gallstone pancreatitis: Significance of distribution of infiltration or fluid collection

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Abstract

AIM: To evaluate the usefulness of various computed tomography (CT) findings including distribution of infiltration or fluid collection in differentiating the major etiologies of acute pancreatitis.

METHODS: We reviewed 75 relatively severe cases of acute pancreatitis of alcoholic ($n = 43$) or biliary stone ($n = 32$) etiology having infiltration or fluid collection on CT. We compared the pancreatic size, CT grading, presence or absence of biliary calculi, and dilatation of pancreatic or bile duct. We also evaluated degree and the distribution of infiltration and fluid collection in each group.

RESULTS: The sizes of pancreas were not different between alcohol group and stone group. Alcohol group showed higher CT grading than stone group ($P < 0.05$). Presence of biliary stone and duct dilatation was statistically significant in differentiating etiology ($P < 0.05$). Alcohol group showed significantly prominent peripancreatic pathology than stone group only in left peritoneal compartment ($P = 0.020$).

CONCLUSION: Alcoholic pancreatitis tends to form more prominent peripancreatic changes than gallstone pancreatitis in relatively severe cases. This is evident on the anterior aspect of left abdomen. Although clinical history and some CT findings usually are a major determinant of the etiology, this pattern of peripancreatic pathology may have an ancillary role in determining the etiologies of acute pancreatitis in the equivocal cases.

INTRODUCTION

Biliary stones and alcohol account for 70%-80% of all acute pancreatitis etiologies^[1]. Differentiation between etiologies is of great importance because it can affect the further diagnostic and therapeutic strategies. Diagnosis and treatment of gallstone pancreatitis require an endoscopic retrograde cholangiopancreatography (ERCP) with sphincterotomy, but it is not warranted in alcohol-induced pancreatitis^[2]. The risks associated with ERCP outweigh any benefit unless the causative biliary stone is present.

Computed tomography (CT) findings associated with gallstone pancreatitis such as presence of stone in the biliary system have been well defined. Clinical information about biliary colic or alcohol abuse is also very useful in diagnosis. Causes of acute pancreatitis are often easily determined. However, some cases lacking medical background or the localization of stones or sludge make cause determination difficult. Although CT findings cannot be a sole determinant of therapy in individual cases, it can play a complementary role especially in indeterminate cases.

The purpose of this study was to evaluate the usefulness of CT findings associated with acute pancreatitis and to differentiate between the two major causes of acute pancreatitis. Special focus was given to the degree and distribution of peripancreatic infiltrations and fluid collection.

MATERIALS AND METHODS

Patients

Abdominal CT scans of 86 patients who had CT diagnosis of acute pancreatitis and were evaluated to have an infiltration or fluid collection in the abdominal cavity were studied retrospectively. All of them could be graded as C, D,

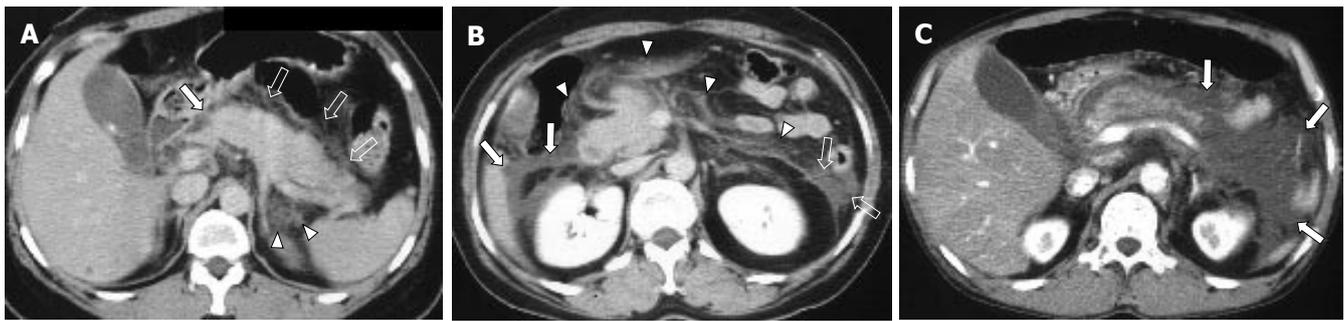


Figure 1 Peripancreatic infiltration scores (1-3). **A:** Score 1 (Irregular peripancreatic infiltration without fluid collection). CT showing irregular strands of peripancreatic fat infiltrations in the compartment of RP (arrow), LP (open arrows), and LSR (arrowhead); **B:** Score 2 (Peripancreatic fluid collection with no or equivocal degree of mass effect). CT showing multiple fluid collections without mass effect in the compartment of RSR (arrows) and LSR (open arrows). Score 1 infiltrations in the compartment of RP and LP (arrowheads) are also seen; **C:** Score 3 (Peripancreatic fluid collection with definite mass effect to adjacent organs). CT showing score 3 fluid collection in the LP and LSR compartments (arrows) displacing or compressing adjacent bowels.

and E by the system suggested by Balthazar *et al.*^[3]. Grade C was defined as pancreatitis with peripancreatic infiltration only. Grades D and E were defined as the disease with single and multiple peripancreatic fluid collections, respectively. Data were not consecutive but extracted randomly from our radiology department CT database. The population was composed of 53 male patients and 33 female patients with a mean age of 56.5 (range, 20-85) years. Patients were divided into an alcohol group and a stone group based upon their clinical diagnosis made by taking into account all possible factors including past medical history such as alcohol abuse or biliary colic, results of radiological studies (ultrasonography, CT, and ERCP), and laboratory data.

Of the 86 patients, eleven were excluded from the study because of unknown causes ($n = 6$), causes other than alcohol abuse or biliary stone ($n = 3$), traumatic pancreatitis ($n = 1$), L-asparaginase-induced pancreatitis ($n = 1$), pancreatitis due to pancreatic divisum ($n = 1$), and cases that were indeterminable between alcoholic and stone pancreatitis ($n = 2$) clinically. The remaining 75 patients were composed of 49 male patients and 26 female patients, with a mean age of 51.3 (range, 27-85) years. Forty-three out of 75 patients (57.3%) were classified as alcohol group, while 32 out of 75 (42.7%) were classified as stone group.

The Institutional Review Board of our hospital did not require approval for retrospective clinical study.

CT protocol

Abdomen CT scans were performed with one of the two helical scanners (Somatom Plus or Somatom Plus 4; Siemens, Erlangen, Germany). Helical CT images of pre-contrast and contrast-enhanced scans were acquired using an 8-mm collimation and 10-mm/s speed (Table 1). Images were reconstructed at an 8-mm interval. A voltage of 120 kVp and amperage of 210 mAs were used. One hundred mL of non-ionic contrast material (Iopromide, Ultravist-300; Schering AG, Berlingen, Germany) was administered intravenously via the antecubital vein at a rate of 3 mL/s with a power injector (OP 100; Medrad, Pittsburgh, PA). Dual-phase contrast-enhancement scan technique was adopted with a delay time of 60-70 s and 200-220 s, respectively, in the majority of cases. Pancreatic

phase scan with a delay time of 30-40 s was available only in a small portion of the patients ($n = 13$) because of lacking of the clinical diagnosis of acute pancreatitis before CT examination. At least one of the two or three phases covered an entire abdominal cavity from diaphragmatic dome to symphysis pubis.

Image analysis

Two abdominal radiologists analyzed the CT findings in consensus. In patients undergoing multiple follow-up CT scans, only the initial study was selected for imaging analysis. The antero-posterior and transverse diameters of pancreatic head, as well as the thickest dimension of body or tail of pancreas were measured. We evaluated the severity of disease at the initial abdominal CT using the CT grading system introduced by Balthazar *et al.*^[3]. We classified them into a three-point-scale, and converted C, D, and E to 1, 2, and 3. We investigated the presence or absence of calculus. Calculus was thought to be present when we saw high attenuation in the biliary tree including gallbladder on pre-contrast scan without measurement of Hounsfield unit. We also examined whether the pancreatic or bile duct was dilated or not. The criteria for abnormal duct dilatation were over 8 mm in diameter for common bile duct and 3 mm in diameter for pancreatic duct.

To assess the distribution of peripancreatic infiltration or fluid collection, the abdominal cavity was divided into 6 compartments, namely right peritoneal (RP) compartment, right superior retroperitoneal (RSR) compartment, right inferior retroperitoneal (RIR) compartment, left peritoneal (LP) compartment, left superior retroperitoneal (LSR) compartment, and left inferior retroperitoneal (LIR) compartment. 'Right' and 'left' compartments were divided by median line of the body traversing the umbilicus and the spinous process of vertebra. 'Peritoneal' and 'retroperitoneal' compartments were divided by already-established anatomical interface between peritoneum and retroperitoneum. Finally, 'superior' and 'inferior' compartments were compartmentalized by the level of lower pole of the left kidney. Degrees of infiltration or fluid collection in each compartment were evaluated with a four-point scale from 0 to 3 (0: no infiltration, 1: irregular infiltrative attenuation without fluid collection, 2: fluid collection with no or equivocal degree of mass effect on

Table 1 CT differentiation of the cause of acute pancreatitis between alcohol and stone group (mean \pm SD)

Findings	Alcohol group (<i>n</i> = 43)	Stone group (<i>n</i> = 32)	<i>P</i>
Size of pancreas			
Anteroposterior diameter, Head	36.2 \pm 6.7 mm	37.3 \pm 6.6 mm	0.485
Transverse diameter, Head	30.7 \pm 5.6 mm	31.7 \pm 5.4 mm	0.434
Thickness, Body and Tail	22.3 \pm 5.2 mm	23.3 \pm 5.3 mm	0.406
CT grading score	2.3 \pm 0.8	1.9 \pm 1.0	0.047 ¹
Calculi in the biliary system	3/43 (7.0%)	22/32 (68.8%)	0.000 ¹
Duct dilatation			
Pancreatic duct	4/43 (9.0%)	10/32 (31.3%)	0.033 ¹
Bile duct	2/43 (4.7%)	19/32 (59.4%)	0.000 ¹

¹Data of statistical significance.

adjacent organ, 3: fluid collection with a definite mass effect on adjacent organ) (Figure 1). If major infiltration or fluid collection of one compartment slightly extended to another compartment, the minor lesion was disregarded. Pancreatic necrosis or pseudocyst was regarded and analyzed as a fluid collection. Infiltration scores of each abdominal compartment in one group were compared with those of the corresponding compartment of the other group. Student *t*-test (pancreatic size), likelihood ratio test (CT grading of acute pancreatitis, grading of infiltration and fluid collection), and Fisher's exact test (calculi and duct dilatation) with 95% confidence interval were used for the evaluation of statistical significance (SPSS for Windows 11.0, Chicago, IL).

RESULTS

The antero-posterior and transverse diameters of pancreatic head for the alcohol and stone group measured 36.2 \pm 6.7 mm \times 30.7 \pm 5.6 mm and 37.3 \pm 6.6 mm \times 31.7 \pm 5.4 mm, respectively. The thickness of pancreatic body or tail in great dimension was 22.3 \pm 5.2 mm and 23.3 \pm 5.3 mm, respectively. Differences in pancreas dimension between the alcohol and stone groups were not significant ($P > 0.05$, Student-*t* test).

Scores of alcohol and stone groups resulting from CT grading (converted from C, D, E to 1, 2, 3), were 2.33 \pm 0.81, and 1.94 \pm 0.95, respectively. Alcohol group showed more aggressive CT findings than stone group ($P = 0.047$, likelihood ratio test).

The alcohol group showed calculi in the biliary tree in 3 of 43 cases (7.0%), while 22 of 32 cases (68.8%) were calculus-positive in the stone group ($P = 0.000$, Fisher's exact test). However, only 5 of 22 stone-positive cases showed calculi in the distal common bile duct. Only 5 of 32 cases (15.6%) in stone group showed distal common bile duct calculi on CT scans. In the remaining cases of stone group, biliary calculi were located in the gallbladder ($n = 11$), intrahepatic duct ($n = 1$), common duct other than distal portion ($n = 3$), and simultaneously in the gallbladder and the common duct other than distal portion ($n = 2$).

Abnormal pancreatic duct dilatation was noted in 4 of 43 cases (9.3%) of alcohol group and in 10 of 32 cases (31.3%) stone group ($P = 0.033$, Fisher's exact test). Bile

Table 2 Comparison of infiltration scores of each abdominal compartment between alcohol group and stone group (mean \pm SD)

Compartment	Alcohol group (<i>n</i> = 43)	Stone group (<i>n</i> = 32)	<i>P</i>
RP	1.00 \pm 0.82	0.91 \pm 0.69	0.568
RSR	0.84 \pm 0.87	0.94 \pm 0.95	0.416
RIR	0.56 \pm 0.85	0.56 \pm 0.84	0.905
Total (right)	2.40 \pm 2.16	2.41 \pm 2.12	0.798
LP	1.67 \pm 0.97	1.00 \pm 0.92	0.020 ¹
LSR	1.49 \pm 0.88	1.13 \pm 0.83	0.119
LIR	0.51 \pm 0.28	0.83 \pm 0.52	0.240
Total (left)	3.67 \pm 2.20	2.41 \pm 1.79	0.153

Abbreviations indicating compartments defined in the text.

¹Data of statistical significance.

duct dilatation was positive in 2 of 43 cases in 19 of 32 cases (59.4%) alcohol group, 59.4% (19/32) of stone group ($P = 0.000$, Fisher's exact test). Abnormal dilatation of either the pancreatic or bile duct had a statistical significance in differentiating between the two groups.

Infiltration scores of each abdominal compartment are summarized in Table 2. The overall degree of peripancreatic infiltration of right abdomen (sum of scores of RP + RSR + RIR) was almost same each other (2.40 \pm 2.16) as in stone group (2.41 \pm 2.12) ($P = 0.798$, likelihood ratio test). However, in the left abdominal compartments (LP + LSR + LIR), stone group (2.41 \pm 1.79) showed a tendency of less peripancreatic infiltration than alcohol group, but not significant (3.67 \pm 2.20) ($P = 0.153$). Among the six abdominal compartments, only the peritoneal aspect of left abdomen (or LP compartment) showed a significant difference in peripancreatic change between the two groups (1.67 \pm 0.97 in alcohol group, 1.00 \pm 0.92 in stone group, $P = 0.020$, likelihood ratio test).

The representative cases of acute pancreatitis caused by biliary stone and alcohol are presented in Figures 2 and 3, respectively.

DISCUSSION

Acute pancreatitis has numerous causes and an obscure pathogenesis. The exact pathogenetic mechanism of acute pancreatitis has not been completely established especially in the field of alcohol-induced pancreatic injury. The basic pathogenesis of acute pancreatitis is pancreatic autodigestion. Premature activation of zymogens within acinar cells, escape of activated enzymes from acinar cells and pancreatic ducts start the autodigestive process. It has not been established how alcohol abuse induces premature zymogen activation and release. However, the mechanism of gallstone pancreatitis is known via animal models. A stone impacted in the ampulla of Vater raises intraductal pressure. Increased pressure makes the pancreatic duct epithelium permeable to molecules of up to 25000 Da. Acute pancreatitis occurs when the pancreatic duct is perfused with active pancreatic enzymes, particularly when microvascular permeability is increased by the actions of histamine or prostaglandins. Thus, pancreatic zymogen activation and increased pancreatic duct permeability may

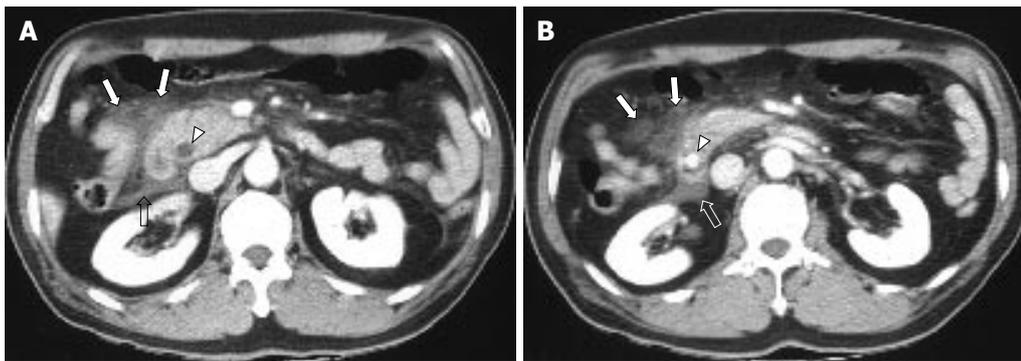


Figure 2 A 61-year old man with a clinical diagnosis of gallstone pancreatitis due to distal common bile duct stone. **A and B:** CT scans showing the peripancreatic infiltration or fluid collection predominantly in the right abdomen: score 1 infiltration in RP compartment (arrows), score 2 fluid collection in RSR compartment (open arrows). Distal common bile duct stone with bile duct dilatation also can be seen (arrowheads).

act sequentially initiating acute pancreatitis^[1,4].

Despite this background knowledge of the pathogenesis of acute pancreatitis, we cannot readily explain why alcohol-induced pancreatitis forms more peripancreatic infiltration or fluid collection than gallstone pancreatitis, especially on the anterior aspect of the left-sided abdomen. As a candidate of the possible explanation for this phenomenon, we assume that the state of intoxication as well as the pain-killing effect of alcohol may play a role in masking acute symptoms of pancreatitis and make patients delay to be hospitalized. On the contrary, biliary stone itself often causes severe pain even before the initiation of acute pancreatitis. However, we cannot explain more prominent involvement of the anterior aspect of the left-sided abdomen by alcohol-induced pancreatitis. This tendency of a more aggressive form of pancreatitis by alcohol-induced disease than stone-associated disease is supported by some previous studies^[5,6].

Most previous efforts to differentiate alcoholic and non-alcoholic pancreatitis have been based on laboratory data or clinical manifestations^[7-10], while there are few studies using imaging modality. The authors of these studies have tried at best, to visualize a stone or bile sludge in the biliary system^[11].

There was a prominent discrepancy between the rate of visible common bile duct stone on pre-contrast CT scan (15.6%) and the rate of common bile duct dilatation (59.4%) in our study. We think that this discrepancy is due to (1) a passed stone with post-inflammatory swelling of ampulla of Vater, (2) a muddy stone in the common bile duct that could not be differentiated from bile on CT scan without measurement of Hounsfield unit, or (3) pancreatic swelling because of acute pancreatitis itself.

Only 15.6% of patients with gallstone pancreatitis showed calculi impacted in the ampulla of Vater although 68.8% were positive for gallstone pancreatitis if the entire biliary tree was included. In other words, 31.2% of gall stone pancreatitis patients did not show any calculi in the biliary system on abdominal CT scans. In most of these cases, diagnosis of gallstone pancreatitis was achieved through other imaging modalities such as ultrasonography or ERCP. Still in a small number of cases, serum bilirubin tests and/or patient history were needed for their clinical diagnosis. Similar situations are not uncommon in daily practice making ancillary CT findings meaningful in the clinical determination of acute pancreatitis etiology.

Based on our analysis, we assumed that the extent

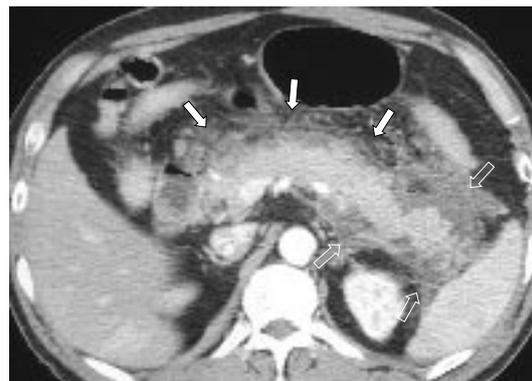


Figure 3 A 57-year old woman with score 1 infiltration in the RP and LP compartments (arrows) and score 2 fluid collections in the LP and LSR compartments (open arrows) is turned out to have a clinical diagnosis of alcoholic pancreatitis.

of the infiltration or fluid collections formed by acute pancreatitis could reflect the severity of the disease, as described by Balthazar *et al*^[5]. Generally, infiltrations or fluid collections of acute pancreatitis tend to involve retroperitoneum rather than peritoneum because the pancreas is located in the retroperitoneal space^[12]. On this point of view, we divided the retroperitoneum into superior and inferior compartments, and we also assumed that, initially, infiltrations might extend to retroperitoneum around pancreas (or superior compartment of retroperitoneum), then simultaneously further inferiorly along the retroperitoneal space and anteriorly to peritoneal space. Therefore, it is necessary to compartmentalize retroperitoneum into superior and inferior and score each compartment independently to weight on the significance of retroperitoneal space as an initially-preferred pathway of disease spread.

Population of our study was confined to the patients who had peripancreatic pathologic findings at initial abdominal CT. Therefore, results from our study cannot be applied to all patients with acute pancreatitis, but should be restricted to the moderate or severe cases in which CT shows peripancreatic pathology. However, we have met relatively severe forms of acute pancreatitis much more frequently in daily CT practice, and these are the real cases needing differentiation of their etiologies. So, we think the results from our study could be helpful.

Pancreatic necrosis is undoubtedly a very important factor for poor prognosis of acute pancreatitis. It has

been proven that patients with necrotizing pancreatitis have much a higher mortality and complication rate than patients with simple pancreatitis^[13]. We regard a pancreatic necrosis as a mere fluid collection because we just want to know the meaning of the extent and distribution of fluid collection in differentiating major etiologies of acute pancreatitis. Whether the fluid is a necrotic pancreatic parenchyma or just a fluid remains unclear. However, we cannot agree that it is a potential limitation of our study.

Other limitations of our study are as follows, (1) Selection of patients was randomized but it was retrospective and not consecutive, possibly introducing potential selection bias; (2) The gold standard grouping of alcoholic and gallstone pancreatitis was the clinical diagnosis using medical records. Improper clinical history taking might play a role in incorrect determination of etiology of acute pancreatitis.

In conclusion, alcoholic pancreatitis tends to form more peripancreatic infiltrations or fluid collections than gallstone pancreatitis. This tendency is more prominent on the anterior (peritoneal) aspect of the left abdomen. Although clinical histories such as biliary colic or alcohol abuse and some CT findings favoring a gallstone pancreatitis usually are a major determinant of the etiology of acute pancreatitis, the degree and distribution of peripancreatic infiltration or fluid collection may have an ancillary role in differentiating the two major etiologies of acute pancreatitis especially in the case of insufficient clinical history or passed-out stone.

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Characterization of pancreatic stem cells derived from adult human pancreas ducts by fluorescence activated cell sorting

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Abstract

AIM: To isolate putative pancreatic stem cells (PSCs) from human adult tissues of pancreas duct using serum-free, conditioned medium. The characterization of surface phenotype of these PSCs was analyzed by flow cytometry. The potential for pancreatic lineage and the capability of β -cell differentiation in these PSCs were evaluated as well.

METHODS: By using serum-free medium supplemented with essential growth factors, we attempted to isolate the putative PSCs which has been reported to express *nestin* and *pdx-1*. The Matrigel™ was employed to evaluate the differential capacity of isolated cells. Dithizone staining, insulin content/secretion measurement, and immunohistochemistry staining were used to monitor the differentiation. Fluorescence activated cell sorting (FACS) was used to detect the phenotypic markers of putative PSCs.

RESULTS: A monolayer of spindle-like cells was culti-

ated. The putative PSCs expressed *pdx-1* and *nestin*. They were also able to differentiate into insulin-, glucagon-, and somatostatin-positive cells. The spectrum of phenotypic markers in PSCs was investigated; a similarity was revealed when using human bone marrow-derived stem cells as the comparative experiment, such as CD29, CD44, CD49, CD50, CD51, CD62E, PDGFR- α , CD73 (SH2), CD81, CD105(SH3).

CONCLUSION: In this study, we successfully isolated PSCs from adult human pancreatic duct by using serum-free medium. These PSCs not only expressed *nestin* and *pdx-1* but also exhibited markers attributable to mesenchymal stem cells. Although work is needed to elucidate the role of these cells, the application of these PSCs might be therapeutic strategies for diabetes mellitus.

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Key words: Putative pancreas stem cell; *Nestin*; *pdx-1*; Phenotypic marker

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<http://www.wjgnet.com/1007-9327/12/4529.asp>

INTRODUCTION

Diabetes mellitus (DM)^[1], one of the global diseases, is the basis for insulin deficiency either due to the inability of insulin secreting β -cells (type I) or insulin resistance (type II) in somatic cells. In the case of type I DM treatment, islets of Langerhan transplantation has been demonstrated to restore normoglycemia^[2]. Nevertheless, prevalent application is still limited by the shortage of donor pancreas, emphasizing the importance of producing β -cells *in vitro* before their transplantation into patients. The putative pancreatic stem cells (PSCs) have been reported in endocrine, acinar, and duct cells of human^[3-5] and mouse studies^[6-8], and the capacity to differentiate pancreatic lineage cells has been demonstrated *in vitro*. However, the existence and the biological role of putative PSCs in β -cell neo-regeneration is still doubtful^[9].

Although the existence of PSCs has been reported in mice and humans, the methodological characterization of these PSCs is still ambiguous. The identification of PSC-specific biomarkers is required not only to define the PSCs operationally, but also to provide an efficient access for further purification. *Nestin*, an intermediate filament first identified in neuroepithelial stem cells, has been maintained as a marker of multi-lineage progenitor cells^[10]. In regeneration studies of pancreas, some *nestin* positive cells have been observed^[5]; *nestin* positive cells isolated from islets^[11,12], mesenchymal cells^[13], pancreatic ducts^[14] and vascular endothelial cells^[15] have been reported. In murine embryonic stem cell (mESC) studies, *nestin* positive cells could be selected and enriched by conventional medium cultivation for further neurogenesis^[16], the application in pancreas was worth contemplating^[17]. Pancreas duodenum homeobox-1 (*pdx-1*) is also essential for pancreatic development, insulin production, and glucose homeostasis. In animal models of partial pancreatectomy^[18], diabetic models of streptozotocin (STZ) treatment in mice^[19], injury and embryology studies^[20,21], the expression of *pdx-1* was detected. For the biological role of the interaction with multiple transcription factors and co-regulators, it was thought as a direct indicator of cells with pancreatic differentiation potentials^[22].

In the present study, we attempted to isolate putative PSCs from adult human pancreatic duct tissue rather than as in previous studies which used the animal model^[13] or the human fetus^[5]. Furthermore, to seek the potential biomarkers on these PSCs, the spectrum of phenotypic markers of human BMSCs was utilized and analyzed. These efforts attempt to investigate the properties of putative PSCs and demonstrate that β -cells could be induced by autogenous pancreatic tissue and possibly apply to diabetes therapy.

MATERIALS AND METHODS

Putative pancreatic stem cells (PSC) isolation

This research follows the tenets and regulations of the Declaration of Helsinki and has been reviewed by the Institutional Review Committee at Taipei Veterans General Hospital. Human pancreatic duct tissues at close proximity to the duct, originating from 4 identical donors, were dissected and digested by collagenase P (Roche Molecular Biochemicals, Mannheim, Germany) with HEPES-buffered saline for 7 h at 37°C. The digested tissue was washed two times with a HBS solution, pipetted up and down several times using a 10 mL syringe with a 22G needle, and placed into 10 cm Petri dishes with 10 mL of CMRL 1066 (5.6 mmol/L glucose, Gibco™, USA) media plus 10 mL/L Fetal bovine serum (FBS, Biological Industries, Israel). After two days incubation a sphere-like floating structure was observed. This suspended cell mass was collected by centrifugation, re-suspended using new serum-free ITSFn medium (composed: 1:1 of DMEM/F12, 0.6 g/L glucose, 25 μ g/mL insulin, 100 μ g/mL transferrin, 20 nmol/L progesterone, 60 μ mol/L putrescine, 30 nmol/L selenium chloride, 2 mmol/L glutamine, 3 mmol/L sodium bicarbonate, 5 mmol/L HEPES buffer, 2 μ g/mL heparin, 20

ng/mL human epidermal growth factor (EGF), 20 ng/mL human basic fibroblastic growth factor (b-FGF) and 20 ng/mL human hepatocyte growth factors, all growth factors were purchased from PerproTech, Israel) and placed into a new dish. The procedure was repeated twice to get rid of non-spherical masses and suspended cells, then the suspended cell mass was transferred to a 6 cm Falcon non-treated cultivation dish for plating, and cultivated using 10mL modified serum-free ITSFn medium. The medium was changed twice and sub-cultured once at a ratio of 1:5 in a week. The proliferation ability of putative PSC cells in passage 5, 10, 15, 20, 25 was examined by doubling time calculation.

Human bone marrow mesenchymal stem cell (BMMSC) isolation

Bone marrow aspirates were taken from the posterior iliac crest of normal adult donors (5 mL each; $n = 4$) and the isolation procedure followed our previous protocol^[23]. Briefly, the bone marrow was washed twice with equal volume of PBS and centrifuged at 300 g for 10 min at room temperature. All washed cells were re-suspended in PBS to 10 mL and nucleated cells were isolated with a Percoll density gradient (diluted with equal volume of 1.073 g/mL Percoll solution, then centrifuged at 900 g for 30 min). The mononuclear cells (MNCs) were then suspended in plates. Expansion medium consisted of Dulbecco's modified Eagle's medium with 1 g/L glucose (DMEM-LG, Gibco) and 10% fetal bovine serum (FBS; Gibco) supplemented with 10 ng/mL bFGF, 10 ng/mL EGF, 10 ng/mL PDGF-BB (R&D), 100 Units/mL penicillin, and 100 μ g/mL streptomycin, and 2 mmol/L L-glutamine (Gibco). All of the nucleated cells were plated in 20 mL medium in a 75 cm² culture dish and incubated at 37°C with 5 ml/L CO₂. After 24-48 h, non-adherent cells were discarded, and adherent cells were thoroughly washed twice with phosphate-buffered saline (PBS). These adherent cells were then cultured through 5 passages and used in flow cytometry studies.

RT-PCR detects the expression of *nestin* and *pdx-1* in putative PSC

Trizol™ (Invitrogen, USA) reagent and GeneStrips™ (RNAure, USA) kits were employed for mRNA purification from putative PSC (passage 5). The Advantage RT-for-PCR Kit (Clontech; BD Biosciences, San Jose, CA) was used to synthesize the first strand of cDNA and 12 μ L of extracted mRNA solution was utilized as the template. The experimental procedure followed the manufacturer's instructions. The sequence of primers used to detect human *nestin*, *pdx-1*, *insulin*, *glucagon* and *somatostatin* expression were as followed: *nestin*, forward: 5'-AGAGGGGAATTCCTGGAG-3', reverse: 5'-CTGAGGACCAGGACTCTCTA-3'; *pdx-1* forward: 5'-CCTTCCCATGGATGAAGTC-3', reverse: 5'-TGTCCCTCCTCTTTTCCAC-3'; *insulin* forward: 5'-CACACCTGGTGGAAGCTCTCT-3', reverse: 5'-GTAGAGGGAGCAGATGCTGGTA-3'; *glucagon* forward: 5'-ATCTGGACTCCAGGCGTGCC-3', reverse: 5'-AGCAATGAATTCCTTGGCAG-3'; *somatostatin* forward: 5'-TTCATCATCTACACGGC-3', reverse: 5'-GAGAG-

TAGAAGCAACCTACC-3'. Amplification was carried out with the program of 94°C for 30 s to denature, 55°C for 30 s for primer annealing and 72°C for 30 s to elongate the PCR product for 30 cycles. The reaction was done on a total volume of 25 μ L containing 0.5 μ mol/L of each primer, 200 μ mol/L dNTP, 2 units of Taq enzyme, and 5 μ L of synthesized cDNA in reaction buffer (500 mmol/L KCl, 100 mmol/L Tris-HCl, pH 8.4, 1.5 mmol/L MgCl₂ and 100 μ g/mL bovine serum albumin).

Pancreatic differentiation by basement-membrane-rich gel (Matrigel™)

The Matrigel™, a commercial preparation of murine basement membrane (BD biosciences, USA), was employed to induce pancreatic differentiation. Briefly, 1×10^4 cells of passage 10 and 20 were suspended with 1 mL medium and were placed on the top of the 6 cm plate coated with Matrigel™ (50 μ L per cm²) and were allowed to gel overnight before additional medium was added. Cell samples were taken for the following Dithizone and immunohistochemistry staining at different time points per week until the end of experimentation (4 wk).

Dithizone staining

Dithizone (DTZ, also named Diphenylthiocarbozone, Sigma, USA), which stains insulin-containing cells bright red, was used to quickly assess the presence of insulin-producing cells. The staining protocol followed was from the study by Shiroy, *et al* 2002^[24].

Immunofluorescent staining

The sphere aggregated by PSC differentiated in Matrigel™ was dug and embedded by O.C.T. (Sakura Finetechnical Co., USA) for frozen section. Sections were fixed by ice-cold acetone (50 mL/L) for 2 min at 4°C, and blocked with 5 g/L skim milk at room temperature for 2 h. The sections were then incubated in rabbit anti-glucagon (1: 500, Abcam, ab11195), rabbit anti-glucagon (1:500, Abcam, ab 930), and mouse anti-insulin (1:100, BioGenex, MU029-UC) antibodies in 5 g/L skim milk at 37°C for 2 h, washed twice by PBS, then followed with secondary antibody incubation (goat anti-mouse IgG with FITC conjugated for insulin detection, Jackson115-095-075, 1:500; goat anti-rabbit IgG with FITC conjugated for glucagon detection, Chemicon AP132F, 1:500; and goat anti-rabbit IgG- TRITC, Chemicon AP132R, 1:500). Specimens were washed in PBS three times after incubating with the secondary antibody and coverslips were applied using Fluoromount-G. The slide plating with HepG2 cells (ATCC) was used as a negative control (data not shown).

Measurement of insulin content/secretion

The differentiated cells from Matrigel™ were washed three times with PBS, and placed in 12-well dishes (Falcon, USA) with RPMI culture medium supplemented with 10 mL/L FBS and adjusted the glucose concentration up to 16 mmol/L (the RPMI contained 5 mmol/L glucose origin) then cultivated 48 h. The supernatant was collected and centrifuged to examine the insulin secretion, and kept at -80°C before use. The attached cells were treated with cold

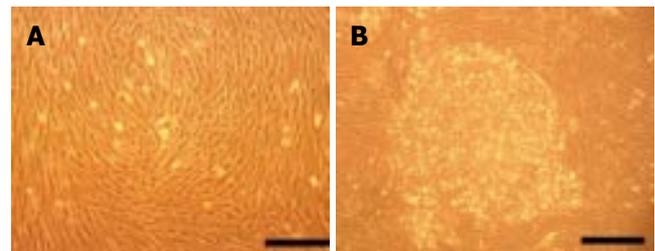


Figure 1 Putative PSC isolated from adult pancreas. **A:** Morphology of cultivated putative PSCs; **B:** Cells aggregate when saturated. bar = 100 μ m.

acid-ethanol (0.1 N hydrochloric acid in absolute ethanol) and kept at 4°C overnight to examine insulin content by ELISA (Mercoxia, Sweden). The clear supernatants were used to investigate the intracellular insulin content and the values obtained were normalized relative to the total protein content (protein assay reagent, Bio-Rad, USA). The RIN-m5F insulinoma cell line (CRL-11605, ATCC) and undifferentiated PSCs were used as controls.

Identification of cell phenotypic markers by FACS

Putative PSC of passage 10 and 20 was used for phenotypic marker identification by FACS. 1×10^5 cells were resuspended in 100 μ L PBS and incubated with primary antibodies at 4°C for 1 h with 1:100 dilutions. After washing twice with PBS, labeled cells were resuspended in 100 μ L PBS with 1 μ L goat anti-mouse IgG conjugated with FITC (Chemicon, AP124F) at 4°C for 1 h, then examined by flow cytometry (BD, USA). The information of antibodies used in investigation was listed in Table 1.

RESULTS

Putative stem cells with nestin expression isolated from adult human pancreas

Putative PSCs with the property of sphere-like cell mass formation were cultivated by a series protocol of isolation and the divergent adhesion to bacteria Petri dishes. Cells with spindle-like shape were observed after plating and served with DMEM/F12 ITSFn serum free medium. The morphological homogeneity of putative PSC was demonstrated (Figure 1A). While the density of culture cells increased, the sphere-like cell aggregation was shown (Figure 1B). The expression of *nestin* and *pdx-1* were detected by RT-PCR, with sustained expression of both genes detected through 20 passages completing this study (RT-PCR examined, Figure 2). The protein level of nestin and *pdx-1* was also examined by immunofluorescent staining (IF) revealing low protein intensity (data not shown). The experimental consistency was illustrated in the repeated examination of tissues from four individual donors.

The pancreatic differentiation potential of putative PSC

The differential competence of putative PSC was inspected by the growth in Matrigel™. The aggregation ability of the isolated PSCs (passage 10) was observed at d 3 after cell seeding (Figure 3A). Color formation was shown when stained with Dithizone at 4-wk after cell seeding, suggest-

Table 1 Comparative analysis of phenotype between human putative PSC and human BMMSC

Cell	Antigen	Donor	Putative PSC								Human BMMSC	Information of antibodies
			Passage 10				Passage 20					
			1	2	3	4	1	2	3	4		
CD29	+	+	+	+	+	+	+	+	+	+	+	abcam, ab8238
CD44	+	+	+	+	+	+	+	+	+	+	+	abcam, ab6337
CD51	+	+	+	+	+	+	+	+	+	+	+	Ancell Corporation, USA
CD81	+	+	+	+	+	+	+	+	+	+	+	BD Pharmingen, clone JS81
SH2 (CD105)	+	+	+	+	+	+	+	+	+	+	+	ATCC, USA
SH3 (CD73)	+	+	+	+	+	+	+	+	+	+	+	ATCC, USA
CD14	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	abcam, ab760
CD38	-	-	-	-	-	-	-	-	-	-	-	abcam, ab1173
CD49b	+	+	+	+	+	+	+	+	+	+	+/-	Ancell Corporation, USA
CD49d	+	+	+	+	+	+	+	+	+	+	+/-	Ancell Corporation, USA
CD50	+	+	+	+	+	+	+	+	+	+	+/-	Ancell Corporation, USA
CD54	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	abcam, ab1048
CD58	+	+	+	+	+	+	+	+	+	+	+/-	abcam, ab1420
CD61	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	abcam, ab7162
CD62E	+	+	+	+	+	+	+	+	+	+	+/-	abcam, ab6630
CD90	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	abcam, ab225
CD109	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	BD Pharmingen, 556039
EGFR	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	abcam, ab30
PDGFR-alpha	+	+	+	+	+	+	+	+	+	+	+/-	R & D systems, USA
CD7	-	-	-	-	-	-	-	-	-	-	-	abcam, ab1249
CD34	-	-	-	-	-	-	-	-	-	-	-	abcam, ab8147
CD45	-	-	-	-	-	-	-	-	-	-	-	abcam, ab6329
CD62P	+	-	+	-	+	-	+	-	+	-	-	abcam, ab6632
CD62L	-	-	-	-	-	-	-	-	-	-	-	abcam, ab222
CD120a	-	-	-	-	-	-	-	-	-	-	-	Serotec, UK
AC133	-	-	-	-	-	-	-	-	-	-	-	Miltenyi Biotec., Germany

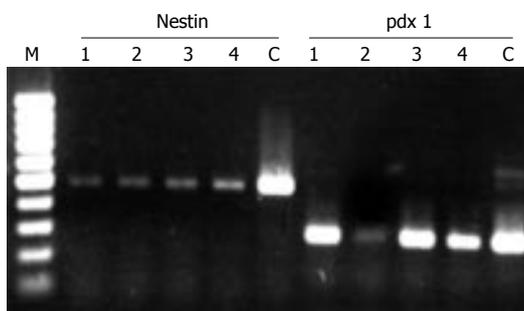


Figure 2 Nestin and pdx-1 expressed in putative PSCs after 5 passages of cultivation. cDNA oriented from 4 individual donors (lane No. 1 to 4), 100 bp marker (M) and the plasmid cloned human nestin and pdx-1 gene with positive control (C) were shown.

ing that cells went through β -cell differentiation in Matrigel™ (Figure 3B).

The expression of *insulin*, *glucagon* and *somatostatin* via RNA level was detectable in the putative PSCs (passage 10) growth in Matrigel™, comparatively; expression was barely illustrated in the group of cells without Matrigel™ (data not shown). Furthermore, the spheroid body of aggregated PSCs showed positive immune reactivity in IF staining (Figures 3 C-E). A similar result was observed in the examination of PSCs after 20 passages, suggesting the differentiation capacity of cells of pancreatic lineage could be preserved.

Measurement of insulin content in differentiated cells

The intracellular insulin content in the differentiating puta-

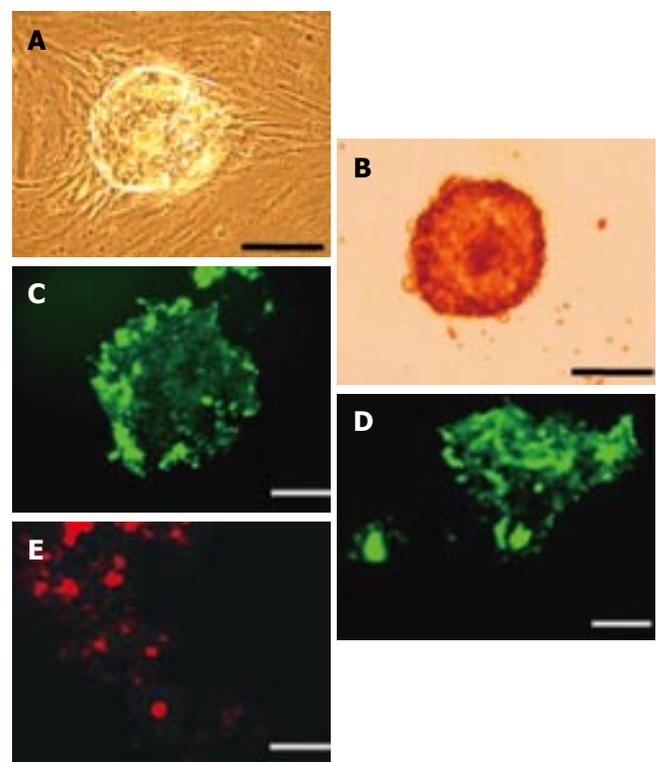


Figure 3 The differentiation of putative PSCs (4 wk). **A:** Growth in Matrigel™; **B:** Dithizone stain; **C-E:** Immunohistochemistry staining by anti-insulin (C), glucagon (D) and somatostatin (E) immunoglobulins. bar = 100 μ m.

tive PSCs (the samples collected from individual donors were pooled together for measurement) were measured at

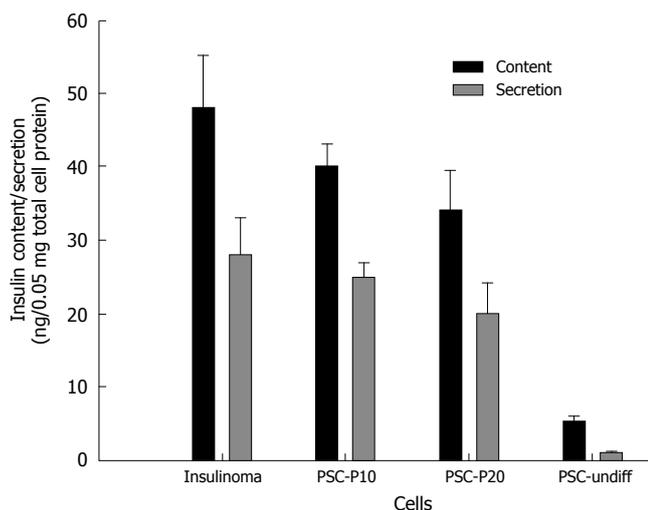


Figure 4 Measurement of Insulin content and secretion in differentiated putative PSCs.

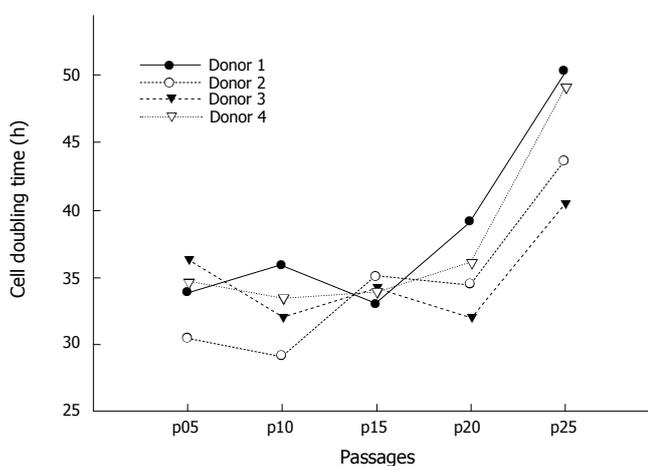


Figure 5 Cell doubling time of putative PSC.

4-wk after cell seeding on Matrigel™ (Figure 4). Compared to the undifferentiated putative PSCs, differentiated PSCs collected at passage 10 and 20 demonstrated the obvious increase in intracellular insulin content/secretion when normalized to the total protein content ($P > 0.01$).

Comparative phenotypic markers spectrum between putative PSC and BMMSC was detected by flow cytometry

To further investigate the potential phenotypic markers in PSCs, the biomarkers that are commonly examined in bone marrow-derived cells were used as the candidates for selection, and the human BMMSC was employed as a comparative study (Table 1). The similar patterns of CD29, CD44, CD51, CD81, SH2 and SH3 were illustrated from both cells. Comparatively, differential intensity was demonstrated on the markers CD38, CD49b, Cd49d, CD50, CD58, CD62E and CDGFR-alpha; the stronger intensity was shown in putative PSCs. The markers of CD62P were revealed only in some PSC but not in BMMSC. No significant difference was shown between the putative PSC batches collected from 4 individual donors,

moreover, the consistency was demonstrated in the studies of putative PSCs of passages 10 and 20.

Cell viability was monitored in long-period cultivation

A simplified cell proliferation method was used to monitor cell viability. The doubling time of 5, 10, 15, 20, 25 passages was illustrated in Figure 5. The cells proliferated rapidly at the beginning (32 h, approximately), however, a remarkable decrease was observed after 20 passages, and failure to proliferate occurred at approximately 30 passages.

DISCUSSION

The existence of putative PSC in pancreatic duct tissue of adult humans was illustrated in the present study. By using serum free medium with essential growth factors, cells with sphere-like mass aggregation were isolated. The putative PSC expressed both *nestin* and *pdx-1*, and these cells were able to differentiate into pancreatic lineage cells that express *insulin*, *glucagon* and *somatostatin* by Matrigel™. The potential biomarkers were evaluated by FACS, and the antibodies that identify common biomarkers of human bone marrow were used. As a comparative study of human BMMSCs, the biomarkers of CD29, CD44, CD51, CD81, SH2 and SH3 (mesenchymal stem cell markers) were all detected on the surface of both cells and higher intensity in PSCs was illustrated in the markers of CD38, CD49b, Cd49d, CD50, CD58, CD62E and CDGFR-alpha. We also found that the expression of CD34, AC 133 (hematopoietic stem cell markers) and CD45 (endothelial marker) were not detected in either PSCs or BMMSCs. The consistency of phenotypic patterns was demonstrated between individual (original cells from 4 identical donors) and the passages (to 20 passages).

Bone marrow (BM)-derived stem cells can be aspirated directly from donors. They can be cultured for *ex vivo* expansion. Previous studies demonstrated the pluripotency of these stem cells. They were able to differentiate into ectodermal^[25,26], endodermal^[26,27], mesodermal^[27], hepatic^[28], cardiac muscle^[29] and skeletal muscle^[30] progenitor cells. By using negative selection, a cell subpopulation isolated from bone marrow, muscle and brain cells have been shown to be able to differentiate into all three germ layers^[31]. This type of mesenchymal stem cells, termed multipotent adult progenitor cells (MAPCs), have the remarkable potential to differentiate not only into mesenchymal cells, but also into cells with visceral mesoderm, neuroectoderm, and endodermal features at the single cell level. Recently, Zhang, *et al.*^[32] demonstrated that the *nestin*-positive progenitor cells isolated from human fetal pancreas, and these cells also have the phenotype markers identical to mesenchymal stem cells (MSCs). In agreement with these results, our results supported these findings and further isolation of *nestin*- and *pdx-1*-positive adult human pancreatic stem cells, which co-expressed the identical MSC markers. These interesting findings also provide evidence to support the interpretation of the study by Ianus *et al.*^[33], that bone marrow derived cells have the capacity to be competently pancreatic islet beta cells. Moreover, the differential capacities and properties of MSCs from different organs are

worth additional attention and may be investigated in a subsequent study.

Nestin is a well-discussed marker of the pancreatic stem cells in embryology, *in vitro* cultivation and pancreatotomy-regeneration in animal models^[1,11,15,32]. However, a contradictory study provided evidence that *nestin*-lineage cells contribute to the microvasculature of pancreas but not endocrine cells of the islet^[1]. The elegant experiment performed by Suzuki *et al*^[3], had clearly demonstrated that the *nestin*-positive cells were isolated from the aggregated insulin-producing precursor and endothelium cells of pancreas by FACS assay^[3]. This result provided a reasonable explanation that the *nestin* antigen is expressed in a much broader precursor population of pancreas, including differentiated cells. The *pdx-1*, compared to the *nestin*, is more specific for pancreatic differentiation^[18-20]. In the present study, the strategy that both the *nestin*- and *pdx-1*- positive cells enriched by serum-free medium was employed followed the previous study of neuronal lineage stem cells isolated from murine embryonic stem cells^[9,16]. Our data supported the claim that the *nestin*- and *pdx-1* expressed stem cells can be isolated from adult pancreatic ducts and possess the differentiation potential of the pancreatic lineage. Moreover, we found that the sustained expression of *nestin* and *pdx-1* in PSCs further exhibited and correlated to the stem cell characteristics of the insulin-producing ability, stable passage and long-term maintenance *in vitro*. Thus, *nestin* and *pdx-1* could not only be the markers of pancreatic stem cells but also play an important role in the self-renewal of beta progenitor cells.

In summary, we use the serum-free method and successfully isolate pancreatic stem cells from adult human pancreatic duct. These PSCs not only expressed the *nestin* and *pdx-1* but also exhibited the markers of mesenchymal stem cells. PSCs and the usage of serum free medium may avoid the potential immune problem of xenogenic protein contamination. Furthermore, this approach should overcome the ethical and immunologic concerns associated with the use of fetal tissues and embryonic stem cells. Although more work is needed to elucidate the role of these PSCs, the application of these PSCs can further be extended and used as an alternative source for therapeutic strategies of diabetes mellitus.

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CLINICAL RESEARCH

A population-based follow-up study on gallstone disease among type 2 diabetics in Kinmen, Taiwan

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no GSD at the first screening, 10 had developed GSD by 2002. The incidence was 3.56% per year (95% CI: 1.78% per year-6.24% per year). Using a Cox regression model, age (RR = 1.07, 95% CI: 1.00-1.14), waist circumference (RR = 1.12, 95% CI: 1.01-1.29), and ALT (RR = 1.13, 95% CI: 1.01-1.26) appeared to be significantly correlated with development of GSD.

CONCLUSION: Older age is a known risk factor for the development of GSD. Our study shows that greater waist circumference and elevated ALT levels are also associated with the development of GSD among type 2 diabetics in Kinmen.

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Key words: Type 2 diabetes; Gallstone disease; Incidence density; Population-based study

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Abstract

AIM: To assess the incidence of and risk factors for gallstone disease (GSD) among type 2 diabetics in Kinmen, Taiwan.

METHODS: A screening program for GSD was performed by two specialists who employed real-time abdominal ultrasound to examine the abdominal region after patients had fasted for at least eight hours. Screening, which was conducted in 2001, involved 848 patients diagnosed with type 2 diabetes. After exclusion of 63 subjects with prevalent GSD, 377 participants without GSD were invited in 2002 for a second round of screening. A total of 281 (74.5%) subjects were re-examined.

RESULTS: Among the 281 type 2 diabetics who had

INTRODUCTION

Gallstone disease (GSD), a digestive disorder with multifactorial origins, is very common worldwide. Within the past few years ultrasonographic studies have provided estimates of GSD prevalence and of predisposing factors in various populations^[1-5]. Although some controversy exists regarding the association between diabetes and GSD, population-based epidemiologic studies have demonstrated that diabetic subjects have an increased morbidity of GSD^[6-8]. Moreover, our previous report showed that the prevalence of overall GSD among type 2 diabetics is higher than in other general Chinese populations when using the same methods for GSD assessment^[5].

Previous study had explored the prevalence of GSD and associated factor among type 2 diabetics^[5], and cross-sectional studies provided useful information of disease prevalence, however, they did not present the incidence or new cases in the study population. One must re-examine the population after a period of time in order to determine incidence and causal relationships between risk factors and

disease. From a preventive medicine viewpoint, primary prevention of GSD should focus on risk factors responsible for the occurrence of GSD. To explore the incidence of and risk factors for GSD is essential to prevent its development and the cholecystectomy caused by this complication, which is often insidious in nature. Therefore, it is necessary to conduct a population-based study which estimates GSD incidence. This is in part due to the fact that more than half of subjects with GSD are unaware of their condition and diagnosed cases seem to represent a selected group based on clinical studies^[8]. Recently, however, a few population-based prospective studies have described the incidence and temporal relationship between the development of GSD and various risk factors among type 2 diabetics in Taiwan. The present study was conducted to explore the incidence and risk factors of GSD among type 2 diabetics in Kinmen, Taiwan based on a one-year follow-up period using real time abdominal ultrasound.

MATERIALS AND METHODS

Organization of gallstone disease screening for type 2 diabetics

Figure 1 shows the procedures for GSD screening between 2001 and 2002. Data used in this study were derived from a population-based screening for type 2 diabetes targeted to subjects aged 30 years or more in Kinmen, Taiwan, between January 1991 and December 1993. The details of the study design and execution have been described in full elsewhere^[9]. The identification of type 2 diabetes was based on the WHO definition in 1985^[10], namely, subjects with a fasting plasma glucose (FPG) ≥ 140 mg/dL or a 2 h postload glucose ≥ 200 mg/dL. Subjects with a history of type 2 diabetes and who had received medication were defined as known cases. However, in the GSD screening done in 2001, even patients who fulfilled the criteria of the revised WHO 1999 were enrolled. That is, additional patients with FPG ≥ 126 mg/dL and <140 mg/dL in 1991 to 1993 were also recruited^[11]. A total of 1123 type 2 diabetics aged 30 and over were identified based on face-to-face interviews carried out by the Yang-Ming Crusade, a volunteer organization of well-trained medical students of National Yang-Ming University. After exclusion of those who migrated or died, the remaining 858 type 2 diabetics formed a cohort to receive first round abdominal ultrasound in 2001. A total of 440 (51.3%) subjects were examined in first screening for GSD. Sixty-three out of 440 type 2 diabetics were diagnosed with GSD. The overall prevalence of GSD was 14.4%, including single stone 8.0% ($n = 35$), multiple stones 3.2% ($n = 14$), and cholecystectomy 3.2% ($n = 14$)^[5]. The 377 diabetics without GSD screened in 2001 were then invited by telephone calls or invitation letters in 2002 to receive a second round of abdominal examinations. Informed consent was obtained from all participants before the GSD screening^[5].

Data collection and diagnosis of gallstone disease

In the present study, fasting blood samples were drawn by public health nurses. Overnight fasting serum and plasma samples (preserved with EDTA and NaF) were collected and kept frozen (-20°C) until analysis for measurements

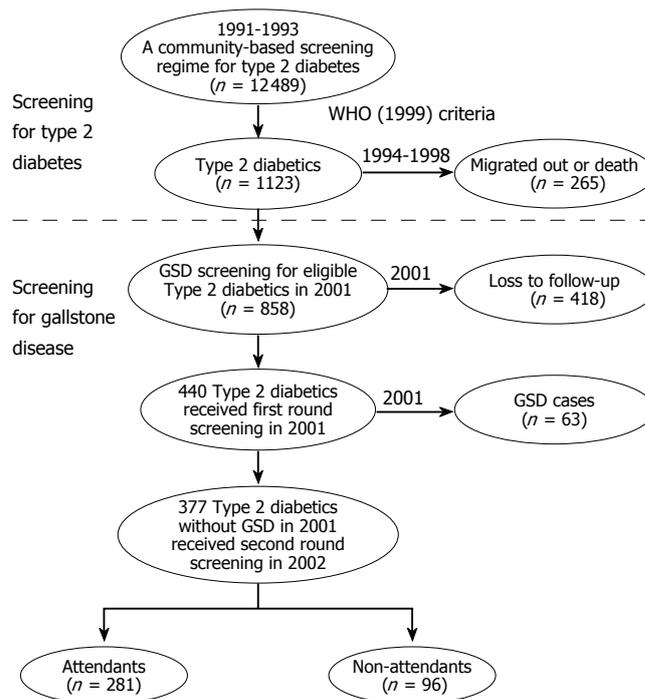


Figure 1 The procedure of screening for gallstone disease among type 2 diabetics during 2001-2002 in Kinmen.

of biochemistry markers. FPG concentrations were determined using the hexokinase-glucose-6-phosphate dehydrogenase method with a glucose (HK) reagent ldt (Gifford, Cberlin, OH). The BMI, waist circumference, uric acid, and HbA_{1c} data were also collected during the GSD screening in 2001. The duration of type 2 diabetes in patients who were previously diagnosed with the disease was confirmed by the questionnaire. In addition, the screening protocol for GSD was performed in 2001 and 2002. GSD was diagnosed by two specialists using real-time abdominal ultrasound to examine the abdominal region after participants had fasted for at least 8 h. GSD was identified based on the presence of “movable hyperechoic material with acoustic shadow. Cases of GSD were classified as follows: single gallbladder stone, multiple gallbladder stones, and cholecystectomy, excluding gallbladder polyps. Cases were identified as any type of GSD among type 2 diabetics.

Inter-observer reliability in ultrasound sonography

In order to set up a consistent diagnosis of GSD among two specialists, the Kappa statistic was used to assess inter-observer reliability among study specialists. A pilot study was performed with a randomly selected cohort ($n = 50$) of type 2 diabetics other than the study subjects. Our pilot study on inter-observer reliability showed a Kappa value for diagnosis of GSD of 0.77 (95%CI: 0.50-0.96).

Statistical analysis

Statistical analysis was performed using SAS software. The incidence of GSD was determined per year based on the ratio of the observed number of cases to the total number of patient-years at risk. Ninety-five percent confidence intervals (95% CI) for incidence were calculated using the Poisson distribution. In the univariate analysis, a *t*-test was

Table 1 Attendance rate of gallstone disease screening among type 2 diabetics in Kinmen

Variable	Eligible population	Screened population	Attendance rate
	<i>n</i>	<i>n</i>	(%)
Sex			
Male	167	121	72.5
Female	210	160	76.2
Age (yr)			
40-49	43	33	76.7
50-59	87	56	64.4
60-69	134	111	82.8
70+	113	81	71.7
Total	377	281	74.5

Table 2 Sex- and age-specific incidence of gallstone disease among type 2 diabetics in Kinmen

Variable	Incidence of any gallstone disease		
	No. of no GSD at first screening	New cases	Incidence density (% per year) (95%CI)
Sex			
Male	121	2	1.65 (0.03-5.10)
Female	160	8	5 (2.29-9.31)
<i>P</i> -value			0.12
Age (yr)			
40-49	33	0	0 (-)
50-59	56	2	3.57 (0.06-11.03)
60-69	111	4	3.6 (1.12-8.37)
70+	81	4	4.94 (1.53-11.47)
<i>P</i> -value			0.04
Total	281	10	3.56 (1.78-6.24)

applied for continuous variables. Multiple Cox regression was used to investigate the independence of factors associated with development of GSD. $P < 0.05$ was considered statistically significant. The results are presented as mean \pm SD.

RESULTS

Of 377 type 2 diabetics without GSD in 2001, 281 subjects attended the second round of abdominal ultrasound examinations in 2002. The overall attendance rate was thus about 74.5%. Subjects were considered as censored cases if the outcomes were not available. Table 1 shows that females had higher attendance rates than males (76.2% versus 72.5%), and old people (50-59 and over 70 year of age) had a slightly lower attendance rate than other age groups.

Table 2 presents gender-specific and age-specific one-year incidences of GSD. Overall incidence of GSD was 3.56% per year (95%CI: 1.78% per year-6.24% per year).

Table 3 Univariate analysis of risk factors for the development of gallstone disease among type 2 diabetics in Kinmen

Variables	Development of gallstone disease			Definitions of disease condition
	Yes Mean \pm SD	No Mean \pm SD	<i>P</i> value for <i>t</i> test	
Age (yr)	69.90 \pm 8.79	63.11 \pm 10.72	0.04	-
Duration of diabetes (yr)	9.60 \pm 0.84	9.44 \pm 1.59	0.59	-
Fasting plasma glucose (mg/dL)	148.50 \pm 18.45	144.94 \pm 39.35	0.58	≥ 126 mg/dL
HbA1c (%)	8.19 \pm 0.93	8.33 \pm 2.05	0.69	$\geq 7\%$
Systolic blood pressure (mmHg)	152.49 \pm 13.91	145.08 \pm 16.35	0.16	≥ 140 mmHg
Diastolic blood pressure (mmHg)	89.55 \pm 9.40	86.74 \pm 9.52	0.36	≥ 90 mmHg
Body mass index (Kg/m ²)	26.57 \pm 1.07	25.36 \pm 2.87	0.01	≥ 27 kg/m ²
Waist circumference (cm)	89.74 \pm 7.95	85.23 \pm 7.52	0.000	≥ 90 cm for males or ≥ 80 cm for females
Total cholesterol (mg/dL)	220.09 \pm 20.57	210.99 \pm 26.82	0.000	≥ 200 mg/dL
Triglyceride (mg/dL)	178.50 \pm 38.95	144.28 \pm 66.72	0.11	≥ 200 mg/dL
AST (U/L)	25.40 \pm 6.95	22.40 \pm 8.15	0.01	≥ 40 U/L
ALT (U/L)	31.36 \pm 9.34	22.39 \pm 10.28	0.01	≥ 40 U/L
Uric acid (mg/dL)	6.07 \pm 1.20	5.94 \pm 1.29	0.76	≥ 7 mg/dL for males or ≥ 6 mg/dL for females

This incidence shows a clear trend with age ($P = 0.04$) with values increasing monotonically: from 0% per year at age 40-49 years to 3.57% per year at age 50-59 years, 3.60% per year at age 60-69 years to 4.94% per year at older ages. There was no consistent pattern by age group. Females had a slightly higher incidence (5.00% per year versus 1.65% per year, $P = 0.12$) than males, although the gender difference was not statistically significant.

Table 3 shows the risk factors for the development of GSD in type 2 diabetics by univariate analysis. The risk factors that were significantly related to the development of GSD included age (t , $T = 1.98$, $P = 0.04$), BMI ($T = 3.18$, $P = 0.01$), waist circumference ($T = 8.16$, $P = 0.0001$), total cholesterol ($T = 4.94$, $P = 0.0001$), AST ($T = 2.83$, $P = 0.01$), and ALT ($T = 2.69$, $P = 0.01$).

To assess the independence of the contributions of these factors to the development of GSD, the significant variables from univariate analysis for GSD were further examined using a Cox regression model including age, BMI, waist circumference, total cholesterol, AST, and ALT. As Table 4 shows, age (RR = 1.07, 95%CI: 1.00-1.14), waist circumference (RR = 1.12, 95%CI: 1.01-1.29), and ALT (RR = 1.13, 95%CI: 1.01-1.26) appeared to be independently correlated with development of GSD.

DISCUSSION

Incidence and risk factors for the development of gallstone disease

Abdominal ultrasound for GSD screening is viewed as a robust method. Previous clinical studies have shown reliable positive (0.99-1.00) and negative (0.90-0.96) predictive

Table 4 Cox regression model of risk factors associated with the development of gallstone disease among type 2 diabetics in Kinmen

Variables	Development of gallstone disease (yes vs no)	
	Relative risk	(95% CI)
Sex (female vs male)	2.60	0.52-13.11
Age (yr)	1.07	1.00-1.14
Body mass index (Kg/m ²)	1.03	0.57-1.34
Waist circumference (cm)	1.12	1.01-1.29
Total cholesterol (mg/dL)	1.01	0.98-1.03
AST (U/L)	0.91	0.77-1.07
ALT (U/L)	1.13	1.01-1.26

values of ultrasonographic diagnosis^[12]. In the present study, the annual incidence of overall GSD was higher than that in other general population-based studies^[12-15], implying that type 2 diabetes might be a positive risk factor for GSD development. Possible pathogenic reasons are that type 2 diabetes combined with GSD might induce acute cholecystitis more often and have a higher probability of progression to septicemia than does gallbladder dysfunction in non-diabetic patients^[16], and late-onset diabetic patients have a higher lithogenic bile index than non-diabetics after adjustment for sex and age^[17]. In addition, hyperglycemia in diabetic subjects might exert effects on gallbladder motility^[18].

An association between GSD and use of exogenous estrogens was confirmed^[19]. The lithogenic effects of estrogen are mediated in part by an increase in bile cholesterol saturation^[19]. However, previous studies showed that cholesterol GSD is common in Western populations whereas pigment GSD is major components in Taiwan^[1]. Unlike result for cholesterol GSD, our results did not show a causal relationship between female sex and development of pigment GSD. The different findings for Orientals from those shown for Occidentals suggest that cholesterol GSD has not yet become a major GSD component in Taiwanese diabetic populations.

Using both univariate analysis and a multiple Cox regression model, our study also demonstrated that age is a significant risk factor for the development of GSD. This result is not concordant with results from other studies that had longer screening intervals^[8,13,15]. Larger amounts of cholesterol secreted by the liver and decreases in the catabolism of cholesterol to bile acid were observed in the elderly^[20]. Although the long-term exposure to many other risk factors in the elderly might account for their increased chance of developing GSD^[5], age still remained a major factor leading to GSD development, irrespective of locality, standard of living, or after adjustment for other demographic and clinical characteristics in the multivariate analysis.

Several population-based studies demonstrated that liver cirrhosis represents a strong risk factor for GSD^[21,22]. The annual incidence of GSD in patients with cirrhosis appears to be about eight times higher than in the general

population^[21]. Alanine aminotransferase (ALT) has, for some time, been viewed as a sensitive indicator of liver-cell injury^[23]. Currently, the determination of serum ALT levels constitutes the most-frequently applied test for the identification of patients suffering from liver disease. This parameter also acts as a surrogate marker for disease severity and/or as an index of hepatic activity^[24]. Our results showing that elevated ALT levels constitute higher risk of GSD development suggest that appropriate integrated diagnosis and therapy in the early stage of liver dysfunction might eventually enable us to prevent incident GSD. Instead of being a sign of more serious liver disease like liver cirrhosis, further studies should be conducted to explore the possibility of whether elevated ALT levels is an indicator for GSD because it indicates a fatty liver (and thus a high BMI), and early stage of chronic liver disease.

Obesity could raise the saturation of bile by increasing biliary secretion of cholesterol, the latter probably depending on a higher synthesis of cholesterol in obese subjects^[25]. Being overweight at baseline was strongly associated with the incidence of GSD which was also suggested in epidemiologic studies^[13]. In this follow-up study, we found that a higher waist circumference rather than a higher BMI was significantly and positively associated with GSD development. Thus abdominal obesity might be more important than BMI for identifying diabetics at high risk of GSD. One possible reason is that due to a high correlation between BMI and waist circumference ($r = 0.64$), waist circumference might explain the effect of BMI on GSD development in type 2 diabetic subjects. Another possible reason is that a large waist circumference might be an unambiguous indicator of excess body fat, except in the presence of abdominal tumors or ascites, and might be a better estimate of overall body fat than is BMI. In addition, from the biological perspective, BMI becomes primarily a surrogate of lean body mass when BMI and height-adjusted waist circumference are included in the same model, because the variation in BMI attributable to adiposity is essentially controlled by the height-adjusted waist circumference variable^[14]. Nevertheless, further epidemiological and etiologic investigations are needed to explore the pathophysiological mechanism underlying gender-related differences in waist circumference and GSD among diabetics.

Methodological considerations

Although using a follow-up study design can clarify the temporal relationship of potential risk factors and the development of GSD, there are some limitations in the present study. First, the characteristics pertinent to the risk of type 2 diabetes for study subjects were not significantly different from non-respondents (except for age), indicating that subjects who did not return for follow-up might have more severe GSD. Also, we assumed that all the new GSD cases occurred in 2002. Since additional GSD cases could occur in subsequent years, the incidence of GSD may be underestimated. Second, all the patients had diabetes in the study population, therefore, an evaluation of the extent of GSD incidence in subjects without diabetes was difficult. Third, we did not attempt to estimate the incidence

of gallstone formation but rather the incidence of newly screened GSD. Our analysis only focused on clinically relevant GSD. Fourth, due to a shorter follow-up period, we did not have a large enough sample size to estimate the “true” effects between potential risk factors and the incidence of GSD. Further long-term studies should be conducted to explore the morbidity of GSD and plausible biological mechanisms underlying its development.

In conclusion, our reports show that the incidence of GSD is 3.56% per year. Significant risk factors for the development of GSD include not only older age, but also, higher waist circumference and elevated ALT levels among type 2 diabetics.

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Decrease of serum carnitine levels in patients with or without gastrointestinal cancer cachexia

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Abstract

AIM: To evaluate the levels of serum carnitine in patients with cancer in digestive organs and to compare them with other cancers in order to provide new insights into the mechanisms of cachexia.

METHODS: Fifty-five cachectic patients with or without gastrointestinal cancer were enrolled in the present study. They underwent routine laboratory investigations, including examination of the levels of various forms of carnitine present in serum (i.e., long-chain acylcarnitine, short-chain acylcarnitine, free carnitine, and total carnitine). These values were compared with those found in 60 cancer patients in good nutritional status as well as with those of 30 healthy control subjects.

RESULTS: When the cachectic patients with gastrointestinal cancer were compared with the cachectic patients without gastrointestinal cancer, the difference was $-6.8 \mu\text{mol/L}$ in free carnitine ($P < 0.005$), $0.04 \mu\text{mol/L}$ in long chain acylcarnitine ($P < 0.05$), $8.7 \mu\text{mol/L}$ in total carnitine ($P < 0.001$). In the cachectic patients with or without gastrointestinal cancer, the difference was $12.2 \mu\text{mol/L}$ in free carnitine ($P < 0.001$), $4.60 \mu\text{mol/L}$ in short chain acylcarnitine ($P < 0.001$), and $0.60 \mu\text{mol/L}$ in long-chain acylcarnitine ($P < 0.005$) and $17.4 \mu\text{mol/L}$ in total carnitine ($P < 0.001$). In the cachectic patients with gastrointestinal cancer and the healthy control subjects, the difference was $15.5 \mu\text{mol/L}$ in free carnitine ($P < 0.001$), $5.2 \mu\text{mol/L}$ in short-chain acylcarnitine ($P < 0.001$), $1.0 \mu\text{mol/L}$ in long chain acylcarnitine ($P < 0.001$), and $21.8 \mu\text{mol/L}$ in total carnitine ($P < 0.001$).

CONCLUSION: Low serum levels of carnitine in terminal neoplastic patients are decreased greatly due to the decreased dietary intake and impaired endogenous

synthesis of this substance. These low serum carnitine levels also contribute to the progression of cachexia in cancer patients.

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Key words: Acetyl carnitine; Nutritional status; Anorexia; Malnutrition; Fatigue

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INTRODUCTION

Cancer cachexia is a condition characterized by anorexia, chronic nausea, early satiety, muscle wasting, involuntary weight loss, lipolysis, weakness, fatigue, impaired mental and physical performance, decreased capacity of wound healing, impaired immunological function, and compromised quality of life. Cancer cachexia is associated with poor survival and decreased tolerance to both radiotherapy and chemotherapy^[1].

The prevalence of cachexia is about 60% in terminal cancer patients^[2]. Anorexia and nausea exacerbate the detrimental effects of tumor-related changes in protein metabolism on nutritional status, leading to increased morbidity and mortality^[3].

The mechanisms responsible for cachexia in cancer patients are poorly understood. One of the factors contributing to weight loss is the reduced food intake, which may be caused by a decrease in appetite or a tumor treatment^[4], mechanical obstruction of the gastrointestinal (GI) tract or intestinal malabsorption.

A decrease in food intake, combined with a decrease in physical exercise, leads to a decline in muscle mass and power.

Several studies have suggested that metabolic control of food intake also exists, in which the biochemical partitioning between fatty acid oxidation and synthesis is a vital signal indicating catabolic or anabolic energy status^[5]. Changes in energy metabolism influence energy intake via energy signals.

Energy signals are independent from body mass, but they inform the brain on the metabolic switch occurring subcellularly between fatty acid oxidation and synthesis^[6]. The combination of reduced energy intake and increased energy expenditure in cancer patients results in substantial weight loss. It has been shown that weight loss in cancer patients comprises both muscle mass and fat mass.

When there is a cancer, several metabolic changes are present in the whole body. It could be possible that one of these factors is represented by a decrease of endogenous synthesis of carnitine or by a reduction of exogenous assumption of carnitine.

Carnitine is a naturally occurring substance required for energy metabolism. In omnivores approximately 75% of carnitine sources come from diet and 25% from endogenous synthesis^[7]. Under normal conditions, omnivores absorb about 70%-80% of dietary carnitine^[8].

Human skeletal muscle, heart, liver, kidney and brain are capable of biosynthesizing carnitine from methionine and lysine to its immediate precursor gamma butyrobetaine^[9].

Carnitine is essential for the transport of long chain fatty acids across the mitochondrial membrane for fatty degradation and energy production and has the ability to shuttle short chain fatty acids from inside the mitochondria to the cytosol.

In a previous study^[10], we observed low serum levels of carnitine in patients with tumoral cachexia, which may be due to a decreased availability of carnitine in the diet or to the altered endogenous biosynthesis.

The aim of the present study was to evaluate the levels of serum carnitine in patients with cancer in digestive organs and to compare them with other cancers in order to provide new insights into the mechanisms of cachexia.

MATERIALS AND METHODS

Fifty-five patients eligible for this study had advanced malignancies localized in various parts of the body. The patients were in the terminal phase of their disease and only palliation was requested. All of them showed a weight loss above 5% in the 6 mo prior to enrollment in the study. Thirty cachectic patients with gastrointestinal cancer (16 males and 14 females, mean age 47.4 ± 7.6 years) had the following diagnoses: cancer of the stomach in 6, cancer of the small bowel in 6, cancer of the colon in 10, cancer of the rectum in 8. Twenty cachectic patients without gastrointestinal cancer (13 males and 12 females, mean age 49.1 ± 6.4 years) had the following diagnoses: lung cancer in 5, renal cell carcinoma in 5, malignant melanoma in 5, bladder cancer in 5, prostate cancer in 2, and breast cancer in 3. Forty of them underwent surgical intervention, while 31 received one or more chemotherapeutic treatments and 6 received radiotherapy. We also enrolled two groups of control subjects.

Sixty cancer patients were in good nutritional status (25 males and 35 females, mean age 48.6 ± 8.4 years). Their diagnoses were as follows: colorectal carcinoma in 10, lung cancer in 15, breast cancer in 10, gastric cancer in 3, bladder cancer in 7, renal cell carcinoma in 4, prostate cancer in 10, and testicular cancer in 1.

Table 1 Characteristics of the study population (mean \pm SD)

Parameter	Cachectic patients with gastrointestinal cancer (30)	Cachectic patients without gastrointestinal cancer (25)	Non cachectic patients with cancer (60)	Healthy subjects (30)
Sex M/F	16/14	13/12	25/35	15/15
Mean age (yr)	47.4 \pm 7.6	49.1 \pm 6.4	48.6 \pm 8.4	51.3 \pm 5.2
Height (cm)	158.9 \pm 6.8	160.6 \pm 7.1	159.4 \pm 7.8	160.2 \pm 6.4
Weight (kg)	46.8 \pm 7.6	51.4 \pm 6.9	60.2 \pm 7.4	63.8 \pm 11.3
SAP (mmHg)	151.8 \pm 12.4	156 \pm 13.2	152.4 \pm 14.7	150.3 \pm 16.8
DAP (mmHg)	84.2 \pm 8.7	86.4 \pm 7.1	80.1 \pm 9.7	80.2 \pm 10.6
Heart Rate bpm	94.2 \pm 9.8	86.2 \pm 11.1	84.2 \pm 7.1	81.2 \pm 10.2

A second control group consisted of 30 healthy individuals aged 51.3 ± 5.2 years. The patient group ($n = 4$) was examined in the morning between 8:00 a.m. and 10:00 a.m. after an overnight fast. Then, venous blood samples were taken and stored in tubes containing ethylenediamine tetra acetic acid (EDTA) or heparin (Table 1).

Serum or plasma was obtained by centrifugation. Serum was analyzed immediately, while plasma and urine were stored at -20°C before analysis. Serum carnitine levels were determined using the Cederblad and Lindstedt method modified by Brass and Hoppel^[7]. Plasma was treated with perchloric acid (final concentration 3% vol: vol) and centrifuged for 2 min at 10000 r/min. Long-chain acylcarnitine was extracted from the pellet after alkaline hydrolysis, while free and short-chain acylcarnitines were extracted from the supernatant. The sum of short-chain acylcarnitine, free carnitine, and long-chain acylcarnitine was considered the serum of total carnitine level.

Creatinine concentrations were determined using a kinetic colorimetric reaction in the same samples used to measure the carnitine concentrations. All laboratory tests were performed using standard laboratory procedures.

All subjects followed a daily diet consisting of 1800 kcal/d with a content of total cholesterol < 300 mg/d, 50% carbohydrates, 20% proteins, and 30% fats (of which 10% were saturated fatty acids, 10% unsaturated fatty acids, and 10% polyunsaturated fatty acids).

Statistical analysis

The results are presented as mean \pm SD. The following two-tailed tests at $P < 0.05$ were used in the study: the Mann-Whitney U-test in the case of two independent samples and the Spearman's rank correlation coefficient test for univariate relationships between variables. In order to evaluate the independent effects of covariates on carnitine concentration, a stepwise multiple linear regression analysis was performed.

Data were analyzed using the statistical package SPSS for Windows 7.5 (SPSS Inc., Chicago, IL, USA).

RESULTS

Comparison of baseline characteristics

The four groups of subjects were comparable in age, height, systolic and diastolic pressure. The difference in weight was 4.50 kg ($P < 0.05$) between cachectic

patients with gastrointestinal cancer and cachectic patients without gastrointestinal cancer, 13.40 kg ($P < 0.001$) between cachectic patients with gastrointestinal cancer and neoplastic non-cachectic patients, and 17.0 kg between cachectic patients with gastrointestinal cancer and healthy subjects ($P < 0.001$). The difference in weight was 8.80 kg ($P < 0.001$) between cachectic patients without gastrointestinal cancer and non-cachectic patients, and 12.40 kg ($P < 0.001$) between cachectic patients without gastrointestinal cancer and healthy subjects.

Comparison of laboratory parameters

When the cachectic patients with gastro-intestinal cancer were compared to the cachectic patients without gastrointestinal cancer, the difference was 12.5 mg/dL in triglycerides ($P < 0.001$), 26.7 IU/L in ALP ($P < 0.001$) and 802.6 ng/mL in CEA ($P < 0.001$).

The difference was 6.8 $\mu\text{mol/L}$ in free carnitine ($P < 0.005$), 0.04 $\mu\text{mol/L}$ in long chain acylcarnitine ($P < 0.05$), 8.7 $\mu\text{mol/L}$ in total carnitine ($P < 0.001$).

When the cachectic patients with gastrointestinal cancer were compared to non cachectic patients with cancer, the difference was 5.8 mg/dL in bun ($P < 0.001$), 7.00 mg/dL in glucose ($P < 0.001$), 34.1 mg/dL in total cholesterol ($P < 0.001$), 31.3 mg/dL in triglycerides ($P < 0.001$), 8.60 IU/L in AST ($P < 0.001$), 45.8 IU/L in ALP ($P < 0.001$) and 815.6 ng/mL in CEA ($P < 0.001$). The difference was 12.2 $\mu\text{mol/L}$ in free carnitine ($P < 0.001$), 4.60 $\mu\text{mol/L}$ in short-chain acylcarnitine ($P < 0.001$), and 0.60 $\mu\text{mol/L}$ in long-chain acylcarnitine ($P < 0.005$) and 17.4 $\mu\text{mol/L}$ in total carnitine ($P < 0.001$).

When the cachectic patients with gastrointestinal cancer were compared to the healthy control subjects, the difference was 27.60 mg/dL in total cholesterol ($P < 0.001$), 25.8 mg/dL in triglycerides ($P < 0.001$), 13.0 IU/L in ALT ($P < 0.001$), 15.60 IU/L in AST ($P < 0.001$), and 76.8 IU/L in ALP ($P < 0.001$) and 845.1 ng/mL in CEA ($P < 0.001$). The difference was 15.5 $\mu\text{mol/L}$ in free carnitine ($P < 0.001$), 5.2 $\mu\text{mol/L}$ in short-chain acylcarnitine ($P < 0.001$), 1.0 $\mu\text{mol/L}$ in long-chain acylcarnitine ($P < 0.001$), and 21.8 $\mu\text{mol/L}$ in total carnitine ($P < 0.001$).

When the cachectic patients without gastrointestinal cancer were compared to the non cachectic patients with cancer, the difference was 3.2 mg/dL in bun ($P < 0.01$), 7.5 mg/dL in glucose ($P < 0.001$), 27.3 mg/dL in total cholesterol ($P < 0.001$), 18.8 mg in triglycerides ($P < 0.001$), 5.9 IU/L in AST ($P < 0.01$), 19.10 in ALP ($P < 0.001$), 5.40 $\mu\text{mol/L}$ in free carnitine ($P < 0.01$), 3.10 $\mu\text{mol/L}$ in short-chain acylcarnitine ($P < 0.005$), 8.7 $\mu\text{mol/L}$ in total carnitine ($P < 0.001$).

When the cachectic patients without gastrointestinal cancer were compared to the healthy controls, the difference was 5.40 mg/dL in Bun ($P < 0.005$), 20.8 mg/dL in total cholesterol ($P < 0.001$), 13.3 mg/dL in triglycerides ($P < 0.001$), 8.7 IU/L in ALT ($P < 0.001$), 12.9 IU/L in AST ($P < 0.001$), 50.1 IU/L in ALP ($P < 0.001$), 8.7 $\mu\text{mol/L}$ in free carnitine ($P < 0.001$), 3.7 $\mu\text{mol/L}$ in short-chain acylcarnitine ($P < 0.01$), 0.6 $\mu\text{mol/L}$ in long-chain acylcarnitine L ($P < 0.005$), 13.1 $\mu\text{mol/L}$ in total carnitine ($P < 0.001$).

Table 2 Laboratory parameters of subjects included in our study (mean \pm SD)

Parameter	Group 1 (30 pts)	Group 2 (25 pts)	Group 3 (60 pts)	Group 4 (30 pts)
Bun (mg/dL)	35.6 \pm 5.4	38.2 \pm 5.1	41.4 \pm 4.7	32.8 \pm 7.9
Glucose (mg/dL)	68.4 \pm 9.1	67.9 \pm 8.7	75.4 \pm 9.1	71.2 \pm 9.4
Creatinine (mg/dL)	0.87 \pm 0.37	0.91 \pm 0.25	1.06 \pm 0.20	0.97 \pm 0.21
Total Cholesterol (mg/dL)	160.1 \pm 13.9	166.9 \pm 14.2	194.2 \pm 8.7	187.7 \pm 10.8
Triglycerides (mg/dL)	144.3 \pm 13.2	156.8 \pm 10.9	175.6 \pm 9.6	170.1 \pm 9.8
ALT (IU/L)	49.7 \pm 9.8	45.4 \pm 7.9	46.1 \pm 7.8	36.7 \pm 4.4
AST (IU/L)	51.4 \pm 8.9	48.7 \pm 7.4	42.8 \pm 9.6	35.8 \pm 6.7
ALP (IU/L)	241.8 \pm 22.6	215.1 \pm 20.7	196 \pm 16.4	165 \pm 18.2
CEA (ng/mL)	847.2 \pm 196.4	44.6 \pm 13.1	31.6 \pm 15.8	2.1 \pm 1.4

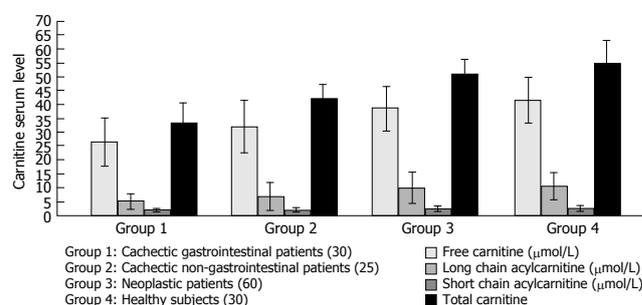


Figure 1 Comparison of carnitine serum levels in four subject groups.

When non-cachectic cancer patients were compared to the healthy controls, the difference was 8.6 mg/dL in BUN ($P < 0.001$), 4.2 mg/dL in glucose ($P < 0.05$), 6.5 mg/dL in total cholesterol ($P < 0.005$), 5.5 mg in triglycerides ($P < 0.01$), 9.4 IU/L in ALT ($P < 0.001$), 7.00 IU/L in AST ($P < 0.001$), and 31.01 IU/L in ALP ($P < 0.001$).

In comparison of serum plasma carnitine in controls and neoplastic patients, there were no significant differences (Table 2).

The correlation studies in patients with gastrointestinal cancer showed a strong correlation between the following parameters: total carnitine ($r = 0.75$), short-chain acylcarnitine ($r = 0.70$), long-chain acylcarnitine ($r = 0.78$), free carnitine ($R = 0.70$), weight loss and total carnitine ($R = 0.81$), short chain acylcarnitine ($r = 0.78$), long chain acylcarnitine ($r = 0.71$), free carnitine ($r = 0.74$) and CEA.

The correlation studies in patients also showed a correlation between total carnitine ($r = 0.61$), short chain acylcarnitine ($r = 0.65$), and long chain acylcarnitine ($r = 0.64$) in free carnitine ($r = 0.61$) and weight loss.

No correlation was found between total carnitine or the fractions of carnitine and other biohumoral and demographic characteristics (Figure 1).

DISCUSSION

The current study demonstrated that patients with gastrointestinal cancer cachexia showed a significant decrease in fractions and total carnitine serum levels in

comparison with controls.

Malignant lesions are generally associated with weight loss, due to delay in the establishment of a diagnosis. Gastrointestinal cancer is characterized by malabsorption with excess fecal loss of ions and water, in addition to nutrient. Cachexia is associated with inflammatory or neoplastic condition that evokes an acute-phase response with an increase of cytokine production (such tumor necrosis factor, interleukin 1, 6 and interferon), and feeding does not reverse the macronutrient changes.

Patients with gastrointestinal cancer may also experience anorexia secondary to food aversion.

Gastrointestinal cancer presents with symptoms and signs such as loss of appetite, abdominal discomfort, decreased gastrointestinal transit with obstructive symptoms, weight loss, weakness, nausea and vomiting.

Diversion to food can be attributed to the location of the tumor, its rate of growth and size^[11].

Patients with growing tumors and poor intake due to bowel obstruction or severe dysphagia present with progressive nutritional deprivation as the main mechanism of cachexia.

The low plasma carnitine concentration in these patients is probably a consequence of decreased intake of carnitine and the carnitine precursors L-lysine and L-methionine.

Carnitine is critical for normal skeletal and heart muscle bioenergetics.

The decrease of carnitine and its derivatives in patients with cachexia can explain the sarcopenia.

In fact, skeletal muscle is the main reservoir in the body and possesses a carnitine concentration at least 50 to 200 times higher than in blood, where the average concentration is about 50 $\mu\text{mol/L}$ ^[12].

Administration of exogenous L- carnitine might improve the nutritional status in patients without gastrointestinal cancer and enhance muscle mass exerting a favorable effect on chronic fatigue syndrome in cancer patients.

The values of serum carnitine determination are questionable in respect to consequences and interpretation. This is due to the fact that serum carnitine represents approximately 3% of total body carnitine.

Rates of changes beyond the limits of 2%-3% in 1 mo can be considered abnormal. However, rates of changes within these limits may also be abnormal if divergent changes are seen in different body compartments (for example, depletion of skeletal muscle plus fluid overload caused by cardiac, hepatic, or renal disease; hypoalbuminemia or intravenous hydration)^[13,14].

Carnitine is required for long-chain fatty acid oxidation and assists in removing accumulated acyl groups from the mitochondria and plays an important role in detoxification^[15]. The reduced levels of carnitine seem to be related with both malabsorption and impaired carnitine production. A decrease in serum carnitine explains not only the sarcopenia but also indicates a bioenergetic deficit with the physical and mental fatigue detectable in patients^[16].

An increase in resting energy expenditure may contribute to weight loss in cancer patients. L-carnitine

has been shown to have physiologic effects on metabolism in cachexia models, presumably because of its ability to increase fatty oxidation^[17].

The decrease in total serum carnitine is associated with a decrease in acylcarnitine, a fundamental substance in brain metabolism. Deficiency in acyl carnitine can induce behavioral and cognitive changes (anxiety, depression and malignancy or treatment-related anorexia)^[18,19].

The direct relationship between total serum carnitine and short-chain and long-chain acylcarnitine levels in patients with gastrointestinal cancer cachexia and CEA suggests that the observed decrease in serum carnitine levels is directly influenced by the activity of gastrointestinal cancer.

In fact, malnutrition triggers a vicious circle in which neoplastic patients produce additional amounts of cytokines. The decrease in total and fractions of carnitine in patients without gastrointestinal cancer cachexia in comparison with cancer patients in good nutritional status and healthy control subjects suggests the alteration of endogenous biosynthesis of carnitine due to elevated cytokine production associated with cachexia. In fact, cachexia has a multifactorial pathogenesis and involves several neuronal systems that modulate production and transport of cell energy, such as hormones, neuropeptides, cytokines and neurotransmitters (serotonin and dopamine)^[20].

Since therapeutic options currently available for the treatment of these patients are not successful,

administration of carnitine may allow us to correct the unregulated immune response and improve the total energy expenditure^[21]. The total energy expenditure involves in fact resting energy expenditure (approximately 70%), voluntary energy expenditure (25%), and energy expenditure in digestion (5%).

Therefore, there is no evidence that supports the use of nutritional or pharmacological intervention to improve the likelihood of either survival or improved anti-neoplastic interventions^[22,23]. L-carnitine and L-acetylcarnitine may be effective at limiting the demands placed on cachectic patients by acute stresses, such as sudden increases in physical activity, immunological challenge or acute and chronic malnutrition.

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RAPID COMMUNICATION

Conserved balance of hepatocyte nuclear DNA content in mononuclear and binuclear hepatocyte populations during the course of chronic viral hepatitis

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Abstract

AIM: To analyze the percentages of hepatocytes with increased nuclear DNA content, i.e., tetraploid (4n) and octoploid (8n) nuclei, and then compared mononuclear and binuclear hepatocyte populations.

METHODS: The percentages of mononuclear diploid (2n), 4n, and 8n hepatocytes and those of binuclear 2 × 2n, 2 × 4n, and 2 × 8n hepatocytes were determined with a method that can simultaneously measure hepatocyte nuclear DNA content and binuclearity in 62 patients with chronic hepatitis B or C. The percentage of 4n and 8n hepatocytes in the mononuclear hepatocyte population was compared with the percentage of 2 × 4n and 2 × 8n hepatocytes in the binuclear hepatocyte population.

RESULTS: The percentages of 4n and 8n hepatocytes in mononuclear hepatocytes and 2 × 4n and 2 × 8n hepatocytes in binuclear hepatocytes were similar, regardless of the activity or fibrosis grade of chronic hepatitis and regardless of the infecting virus.

CONCLUSION: The distribution of nuclear DNA content within mononuclear and binuclear hepatocyte populations was conserved during the course of chronic viral hepatitis.

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Key words: Chronic viral hepatitis; Hepatocyte binuclearity; Hepatocyte ploidy; Nuclear DNA content

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INTRODUCTION

Hepatocyte polyploidization and binucleation are two important features of liver growth and physiology. In adult humans, the number of polyploid liver cells is reportedly 20%-45%^[1,2]. The importance of controlling hepatocyte polyploidization in both normal and pathological liver conditions has been reported. During postnatal growth, the liver parenchyma undergoes dramatic changes characterized by gradual polyploidization^[3]. In adults, hepatocyte polyploidization is differentially regulated upon loss of liver mass and liver damage. Partial hepatectomy induces marked cell proliferation followed by an increase in hepatocyte ploidy^[4]. In contrast, in different liver pathologies, such as hepatocellular carcinoma, growth shifts to a non-polyploidizing pattern and expansion of the diploid hepatocyte population is observed^[5].

We previously studied changes in hepatocyte ploidy and binuclearity profiles in patients with chronic viral hepatitis and found that the percentage of diploid hepatocytes was significantly reduced in patients with high hepatitis activity and marked fibrosis and that the polyploid hepatocyte fraction was increased in these patients^[6]. Thus, during chronic hepatitis, the changes in ploidy is similar to that observed after partial hepatectomy in adults. In this previous study, we validated a technique that allows simultaneous measurement of hepatocyte nuclear DNA content and hepatocyte binuclearity in the same liver sections. This is a technique that makes it possible to evaluate the DNA content of a binuclear hepatocyte.

In the present study, we used the same technique to analyze the nuclear DNA contents of mononuclear and binuclear hepatocytes, to compare the changes in the distribution of hepatocytes with different nuclear DNA content between mononuclear and binuclear hepatocyte populations during the course of human chronic hepatitis.

MATERIALS AND METHODS

Subjects

A total of 62 patients with chronic viral hepatitis (44 men

and 18 women, mean age 39.6 ± 5.7 years) were studied. An ultrasonography-guided needle liver biopsy had been performed on all patients between March 1996 and March 2001. Twenty-seven patients were chronically infected with hepatitis B virus (HBV), whereas the other 35 patients were infected with hepatitis C virus (HCV). On the basis of histological findings, hepatitis activity as determined with the METAVIR scoring system^[7] was A0 in 15 patients, A1 in 13 patients, A2 in 13 patients, and A3 in 21 patients, and the degree of fibrosis was F0 in 7 patients, F1 in 21 patients, F2 in 11 patients, and F3 in 23 patients.

Methods

Analyses of hepatocyte nuclear DNA content and hepatocyte binuclearity were performed as described previously^[6,8,9]. Three-micrometer-thick, paraffin-embedded liver tissue sections were incubated for 1 h with anti-cytokeratin antibody (1:50, KL1, Immunotech, S.A., Marseille, France) as the primary antibody for immunostaining of hepatocyte membrane, followed by a 30-min incubation with biotinylated swine anti-goat, mouse, rabbit immunoglobulin solution (1:200, Dako) and a 30-min incubation with FITC-conjugated streptavidin (1:200, Dako). Sections were then stained for 20 min with Hoechst 33342 (1 mg/L) to stain DNA. Tissue sections were then examined under a Zeiss inverted microscope (Axiovert 35, Carl Zeiss, Gottingen, Germany) equipped for epi-illumination. Images were captured with a cooled, charged coupled device (CCD) camera (KAF 1400-G2, class 2, Photometrics, Tucson, AZ) on 4056 grey levels. Automatic quantitative image analysis was performed in 12 bits with the IPLab Spectrum version 3.1 software. Hepatocytes were classified as mono- or binuclear hepatocytes on the basis of comparisons of fluorescent and membrane labelling images. Other liver cell types, which were defined by morphologic characteristics, were eliminated. Integrated fluorescence intensity was stored in computer files for analysis. A histogram of the fluorescence intensity per section was drawn, and the distribution of each hepatocyte population (2n [diploid] DNA content as the first peak on the histogram, 4n [tetraploid] DNA content as the second peak on the histogram, and 8n [octoploid] DNA content as the third peak on the histogram) was calculated. To develop a histogram of the fluorescence intensity of binuclear hepatocytes, we used the fluorescence intensity of the nucleus with the clearest edge when only one of the two nuclei of a binuclear hepatocyte had a clear edge on the image analyzed. When two nuclei had clear edges, we calculated the average of fluorescence intensity of the two nuclei and used this value as fluorescence intensity of the hepatocyte. A minimum of 300 hepatocytes on eight to 12 separate fields was studied. To ensure that the same cells were not counted twice, slides were read by two observers in a systematic manner of moving from right to left along the slide and then along successive descending lines. All analyses were performed blind to any sample-specific data.

Correlations between values were analyzed by Spearman test, and a $P < 0.05$ was accepted as statistically significant. The study was approved by the Institutional Review Board of Hopital Necker-Enfants Malades (Paris, France) and was carried out in accordance with the Helsinki declaration.

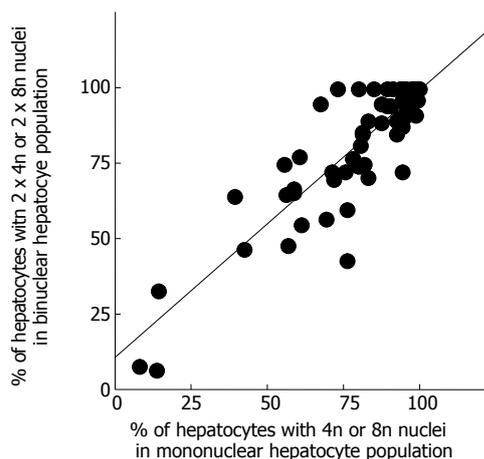


Figure 1 Correlation between percentage of hepatocytes with 4n or 8n nuclei in mononuclear hepatocyte population and percentage of hepatocytes with 2 x 4n or 2 x 8n nuclei in binuclear hepatocyte population. [$P < 0.0001$; correlation coefficient, 0.826 (0.726-0.892)].

RESULTS

We analyzed the percentages of binuclear hepatocytes with diploid (2n) nuclei and those with tetraploid (4n) or octoploid (8n) nuclei (namely binuclear $2 \times 2n$ and $2 \times 4n + 2 \times 8n$) and compared them with the percentages of mononuclear hepatocyte with 2n nucleus and those with 4n or 8n nucleus (namely mononuclear 2n and $4n + 8n$) in samples from patients with chronic viral hepatitis. We found a significant correlation between the percentages of mononuclear 4n and 8n hepatocytes and binuclear $2 \times 4n$ and $2 \times 8n$ hepatocytes ($P < 0.0001$; correlation coefficient, 0.826 [0.726-0.892]); the percentages of nuclei of 4n or 8n DNA content was maintained in the mononuclear hepatocyte population and in the binuclear hepatocyte population (Figure 1). This correlation was maintained when we focused specifically on patients with HBV or HCV. The correlation was also maintained regardless of activity of hepatitis, grade of liver fibrosis, age, or sex (data not shown). In contrast, there was no correlation between the percentage of binuclear $2 \times 4n$ and $2 \times 8n$ hepatocytes and the percentage of binuclear hepatocytes in the total hepatocyte population ($P = 0.1276$; correlation coefficient, 0.196 [-0.057-0.425]; Figure 2). The percentage of binuclear hepatocytes with increased nuclear DNA content ($2 \times 4n$ and $2 \times 8n$ hepatocytes), therefore, was not associated with the percentage of binuclear hepatocytes in the total hepatocyte population.

DISCUSSION

Flow cytometry can measure nuclear DNA content but cannot distinguish between nuclei of mononuclear hepatocytes and those of binuclear hepatocytes. In addition, morphologic observation of hematoxylin and eosin-stained sections can distinguish mononuclear and binuclear hepatocytes but cannot quantify nuclear DNA content. To our knowledge, the present study is the first to compare the distributions of nuclear DNA content between mononuclear and binuclear hepatocyte populations with a novel method that allowed evaluation of the distribution of nuclear DNA content ($2 \times$

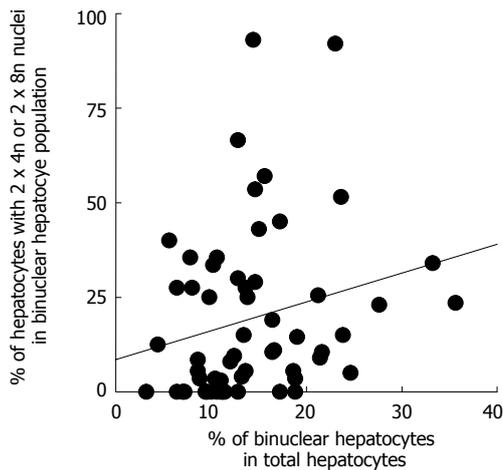


Figure 2 Correlation between percentage of hepatocytes with $2 \times 4n$ or $2 \times 8n$ nuclei in binuclear hepatocyte population and that of binuclear hepatocytes as a portion of total hepatocytes. [$P = 0.1276$; correlation coefficient, $0.196 (-0.057-0.425)$].

$2n$, $2 \times 4n$, and $2 \times 8n$) exclusively in binuclear hepatocytes.

With respect to the hypothesis that mononuclear hepatocytes are stimulated to form binuclear hepatocytes, our findings suggest that both diploid and polyploid mononuclear hepatocytes are similarly stimulated to form binuclear hepatocytes, suggesting the possibility that the formation of binuclear hepatocytes is regulated by a single factor for both diploid and polyploid hepatocytes. Alternatively, it is possible that both diploid and polyploid hepatocytes are sensitive to a single stimulus for binuclear hepatocyte formation. This appears to be independent of viral infection and is maintained throughout the course of chronic hepatitis.

In a previous report^[6], we showed that the decrease in the percentage of diploid hepatocytes was correlated with the progression of chronic hepatitis. In addition, we showed there was a higher percentage of binuclear hepatocytes in patients with HBV infection than those with HCV infection. Our present results indicate that the percentage of diploid hepatocytes decreases in both mononuclear hepatocyte and binuclear hepatocyte populations, in a similar way according to the progression of chronic hepatitis. Also, the present data indicate that binuclear hepatocytes increase in patients infected with HBV, conserving the distribution of different nuclear DNA content ($2 \times 2n$, $2 \times 4n$, and $2 \times 8n$ hepatocytes).

The mechanisms that underlie hepatocyte polyploidiza-

tion are still largely unknown. The importance of binuclear hepatocyte formation as a step towards hepatocyte polyploidization has been reported. We previously provided direct evidence for the pivotal role of binuclear hepatocytes in the formation of $4n$ mononuclear hepatocytes, indicating a close association between binuclear hepatocyte formation and polyploid mononuclear hepatocyte formation *in vitro*^[9]. Our present study indicated that formation of polyploid mononuclear hepatocytes is associated with formation of polyploid binuclear hepatocyte in human chronic viral hepatitis. Further studies are needed to clarify the mechanisms that control hepatocyte polyploidization and binucleation during human chronic viral hepatitis. In addition, the influence of steatosis, which is often observed in case of chronic hepatitis C and can reduce replication in hepatocytes, on hepatocyte polyploidization and binucleation should be elucidated in the future.

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Functional activity of the rectum: A conduit organ or a storage organ or both?

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Abstract

AIM: To investigate whether the degree of rectal distension could define the rectum functions as a conduit or reservoir.

METHODS: Response of the rectal and anal pressure to 2 types of rectal balloon distension, rapid voluminous and slow gradual distention, was recorded in 21 healthy volunteers (12 men, 9 women, age 41.7 ± 10.6 years). The test was repeated with sphincteric squeeze on urgent sensation.

RESULTS: Rapid voluminous rectal distension resulted in a significant rectal pressure increase ($P < 0.001$), an anal pressure decline ($P < 0.05$) and balloon expulsion. The subjects felt urgent sensation but did not feel the 1st rectal sensation. On urgent sensation, anal squeeze caused a significant rectal pressure decrease ($P < 0.001$) and urgency disappearance. Slow incremental rectal filling drew a rectometrogram with a "tone" limb representing a gradual rectal pressure increase during rectal filling, and an "evacuation limb" representing a sharp pressure increase during balloon expulsion. The curve recorded both the 1st rectal sensation and the urgent sensation.

CONCLUSION: The rectum has apparently two functions: transportation (conduit) and storage, both depending on the degree of rectal filling. If the fecal material received by the rectum is small, it is stored in the rectum until a big volume is reached that can affect a degree of rectal distension sufficient to initiate the defecation reflex. Large volume rectal distension evokes directly the rectoanal inhibitory reflex with a resulting defecation.

INTRODUCTION

The rectum, a continuation of the colon, acts to propel the stools to the exterior and is thus considered as a conduit^[1-3]. However, its function as a conduit for stools is controversial^[4-6]. Stools are palpable on digital rectal examination (DRE) in many healthy volunteers who do not feel the desire to defecate^[4-6]. Neil and Rampton^[5] reported that stools are present in the rectum in 31% of normal subjects. Stools are also palpated by DRE in the lower rectum of healthy subjects and appear in the radiograms. Our studies have shown that the rectum stores the stools if the quantity is small, while functions as a conduit if the stools received from the sigmoid colon are voluminous. A small stool volume reaching the rectum from the sigmoid colon probably does not affect rectal distension or evoke the rectoanal inhibitory reflex (RAIR). In such case the stools accumulate in the rectum until they reach a big distending volume that initiates the RAIR. On the other hand, big distending volumes of the stools propelled from the sigmoid colon appear to be able to initiate the RAIR and expel the stools without storage in the rectum. Accordingly, the rectal function as a storage organ or a conduit appears to depend on the volume of fecal matter passing from the sigmoid colon to the rectum and consequently on the degree of rectal distension the volume creates.

We thus hypothesized that rectal function as a conduit or reservoir would depend on the degree of rectal distension. This hypothesis was investigated in the current study.

MATERIALS AND METHODS

Subjects

Twenty-one healthy volunteers (12 men, 9 women, mean

age 41.7 ± 10.6 years, range 29-56 years) participated in the study. They were fully informed about the nature of the study, the tests to be done, and their role in the study. They had no anorectal complaint either in the past or at the time of enrolment. The mean stool frequency was 8.6 ± 1.2 times per week (range 8-11), being in accord with that of normal volunteers in our laboratory. Physical examination, including neurological assessment, was normal. Laboratory work was unremarkable. Sigmoidoscopy assured that the rectum was normal in all the subjects. The subjects gave an informed consent, and the study was approved by the Review Board and Ethics Committee of the Cairo University Faculty of Medicine.

Methods

The subject was instructed to fast for 12 h before the test, and the bowel was evacuated by saline enema. The rectum was distended by a thin polyethylene infinitely compliant balloon, 3 cm in diameter, which was attached to the end of a 10 F tube (London Rubber Industries Ltd, London, UK). The pressure measured within the balloon was considered to be representative of the rectal pressure. With the subject lying in the left lateral position and under no medication, the collapsed balloon was introduced into the rectum through the anus. The tube was connected to a strain gauge pressure transducer (Statham 23 bb, Oxnard, CA).

The anal and rectal pressures were separately measured with a saline-perfused tube. The 10 F tube with multiple side ports at its distal closed end was introduced through the anus for 2-3 cm to lie in the rectal neck (anal canal). Another tube was introduced for 8-10 cm to lie in the rectum. The tube was connected to a pneumohydraulic capillary infusion system (Arndorfer Medical Specialities, Greendale, WI), supplied with a pump that delivers saline solution continually via the capillary tube at a rate of 0.6 mL/min. The transducer outputs were registered on a rectilinear recorder (RS-3400, Gould Inc, Cleveland, OH). Occlusion of the recording orifice produced a pressure elevation of greater than 250 cm H₂O/s.

Prior to anal and rectal pressure recording, the gut was allowed a 20-min period to adapt to the rectal balloon and the manometric tubes in the anal canal and rectum. We recorded the response of the rectal and anal pressures to 2 types of rectal balloon distension: rapid voluminous distension and slow gradual distension. The rectal balloon was filled with 200 mL of normal saline in 10 s for rapid filling, and with increments of 20 mL in 10 s for slow filling. The test was performed at rest and while the anal sphincters were squeezed. The subjects were asked to report the onset of awareness of "something" in the rectum (the first rectal sensation) and the urgent sensation to defecate.

To ensure reproducibility of the results, the tests were repeated at least twice in the individual subjects and the mean value was calculated. The results were analyzed statistically by the Student's *t* test and the values were given as mean \pm SD.

RESULTS

The study was completed in all the subjects with no

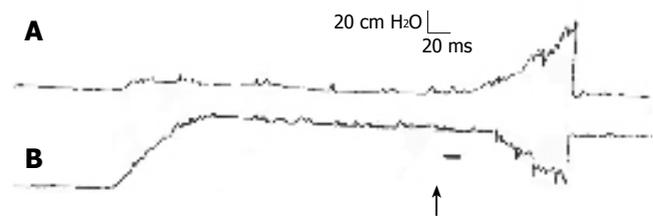


Figure 1 Pressure tracing showing the rectal (A) and anal (B) pressure response to rapid balloon rectal distension with 200 mL of normal saline in 10 seconds. \uparrow = rectal balloon distension.

adverse side effects. The mean resting rectal pressure was 8.7 ± 0.9 cm H₂O (range 8-10) and the mean anal pressure was 73.6 ± 4.6 cm H₂O (range 65-79).

Rectal and anal pressure response to rapid voluminous rectal distension

Rectal balloon distension with 200 mL of normal saline in 10 s resulted in a significant increase of the rectal pressure and a decrease of the anal pressure (Figure 1). The balloon was expelled to the exterior. The mean rectal pressure was 63.4 ± 8.2 cm H₂O (range 56-82 cm H₂O, $P < 0.001$) and the mean anal pressure was 22.4 ± 2.6 cm H₂O (range 18-26 cm H₂O, $P < 0.01$). The subjects felt the urgent sensation followed by balloon expulsion, but did not feel the first rectal sensation. When the subject upon feeling the urgent sensation, was asked to squeeze the anal sphincters for seconds, the mean anal pressure rose to 146.8 ± 16.2 cm H₂O (range 128-166 cm H₂O), resulting in disappearance of the urgent sensation and a significant decrease in the rectal pressure to 10.8 ± 1.1 cm H₂O (range 8-12 cm H₂O, $P < 0.001$). The balloon was not expelled but stored in the rectum. After a few seconds however, if the patient was asked not to squeeze the sphincters, the urgent sensation recurred and the balloon was expelled.

The test was reproducible with no significant difference ($P < 0.05$) in the recorded pressure values. There was also no significant difference between men and women.

Rectal and anal pressure response to slow incremental rectal filling

During rectal filling with a small volume (20 mL in 10 s), rectometrograms were produced (Figure 2). The curve showed a "tone limb" representing the rectal pressure as it increased during rectal filling, and an "evacuation limb" representing the rectal pressure during the process of balloon expulsion. The tone limb carried the points of incidence of the first rectal and urgent sensations while displaying a gradual incline until expulsion started.

The evacuation limb described a "curve" which was continuous with the tone limb. It was manifested at a variable distance after the urgent sensation and depended on the subject's desire to evacuate. The evacuation curve had an ascending limb rising slowly as a continuation of the tone limb, and a vertical descending limb (Figure 2).

The mean rectal balloon distending volume was 78.3 ± 16.6 mL (range 53-108 mL) at the first sensation and 152.6 ± 18.9 mL (range 106-182 mL) at the urgent sensation. The mean rectal pressure was 51.7 ± 9.8 cm H₂O at the 1st rectal sensation and 62.9 ± 14.2 cm H₂O at the urgent

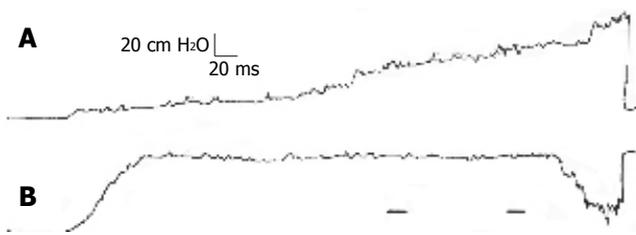


Figure 2 Pressure tracing (rectometrograms) showing the rectal (A) and anal (B) pressure response to balloon rectal distension in increments of 20 mL in 10 seconds.

sensation, while the mean anal pressure was 70.6 ± 5.2 cm H₂O and 18.7 ± 2.3 cm H₂O, respectively (Table 1).

The aforementioned results were reproducible with no significant difference when the test was repeated in the individual subjects.

DISCUSSION

The rectum presumably receives stools from the sigmoid colon, either in a big volume affecting rapid voluminous distension of the rectum, or in small masses affecting gradual rectal distension. The current study could shed some light on the rectal function during the rapid voluminous distension and the slow gradual rectal distension.

Rectum as a conduit

Rapid voluminous rectal distension seems to evoke the recto-anal inhibitory reflex as is evident from the increased rectal and decreased anal pressures as well as from balloon expulsion. The subjects feel the urgent sensation but not the first rectal sensation which is normally felt in the gradual rectal distension. In such conditions, the rectum is considered as a conduit, conducting the stools directly from the colon to the exterior. It is likely that the big voluminous rectal distension induces stimulation of the rectal mechanoreceptors with a resulting initiation of the defecation reflex. In this case the rectum evacuates the received stools and remains empty, provided the circumstances are opportune for defecation.

Squeeze of the anal sphincters could abort the urgent sensation and affect waning of the desire to defecate. The mechanism needs to be further discussed. If circumstances are inopportune for defecation, sphincteric squeeze causes rectal relaxation mediated by the voluntary anorectal inhibition reflex^[7]. However, after some time, the loaded rectum re-contracts probably due to re-stimulation of the rectal mechanoreceptors. If the desire to defecate is still opposed, the external sphincter contracts again. This process is repeated till either defecation is acceded to, or a prolonged contraction of the un-striped rectal detrusor tires out the striped short-contracting external anal sphincter which involuntarily relaxes leading to internal anal sphincter relaxation and opening of the rectal neck.

Rectum as a reservoir

Slow incremental rectal filling does not affect rectal

Table 1 Anal and rectal pressures at the 1st rectal sensation and urgent sensation on slow gradual rectal filling (mean \pm SD)

Pressure (cm H ₂ O)	Rectal		Anal	
	Mean	Range	Mean	Range
Basal	8.7 \pm 0.9	8-10	73.6 \pm 4.6	65-79
1 st rectal sensation	51.4 \pm 9.8 ^d	32-68	70.6 \pm 5.2	61-78
Urge	62.9 \pm 14.2 ^d	45-86	18.7 \pm 2.3 ^b	16-24

^b*P* < 0.01 vs the basal values; ^d*P* < 0.001 vs the basal values.

contraction until rectal filling reaches a large enough volume to stimulate the rectal mechanoreceptors and evoke the defecation reflex. During the period of slow gradual filling, the rectum presumably contains stools which are commonly palpated in some subjects during DRE. We postulate that in such cases the amount of stools received from the sigmoid colon is too small to stimulate the rectal mechanoreceptors or evoke the defecation reflex. In such conditions, the stools may remain accumulated in the rectum for an extended period during which the rectum acts as a “storage” organ. We speculate that stool storage in the rectum may affect its consistency and act as a contributing factor in the genesis of constipation. The storage function of the rectum is apparently due to the adaptability of its smooth muscle to small volume rectal distension. This mechanism of “receptive relaxation” is similar to that occurring in the stomach.

In conclusion, the rectum has apparently 2 functions: transportation (conduit) and storage, both depending on the degree of rectal distension. If the fecal material received by the rectum is small, it is stored in the rectum until a big volume accumulated affects rectal distension sufficient to initiate the defecation reflex. However, stool storage in the rectum may be a contributing factor in the genesis of constipation. Large volume rectal distension evokes directly the recto-anal inhibitory reflex with a resulting defecation.

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Azithromycin in one week quadruple therapy for *H pylori* eradication in Iran

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Abstract

AIM: To investigate eradication rates, patient compliance and tolerability of a 1-wk Azithromycin-based quadruple therapy versus the 2-wk conventional therapy.

METHODS: A total of 129 *H pylori*-positive patients were randomized to either omeprazole 20 mg, bismuth subcitrate 240 mg, azithromycin 250 mg, and metronidazole 500 mg, all twice daily for 1-wk (B-OAzM) or omeprazole 20 mg, bismuth subcitrate 240 mg, amoxicillin 1g, and metronidazole 500 mg all twice daily for 2-wk (B-OAM). *H pylori* infection was defined at entry by histology and rapid urease test and cure of infection was determined by negative urea breath test.

RESULTS: *H pylori* eradication rates produced by B-OAzM and B-OAM were 74.1% and 70.4% respectively based on an intention to treat analysis, and 78.1% versus 75.7% respectively based on a per-protocol analysis. The incidence of poor compliance was lower, although not significantly so, in patients randomized to B-OAzM than for B-OAM (3.5% versus 4.3%) but intolerability was similar in the two groups (35% versus 33.3%).

CONCLUSION: 1-wk azithromycin based quadruple regimen achieves an *H pylori* eradication rate comparable to that of standard 2-wk quadruple therapy, and is associated with comparable patient compliance and complications.

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Key words: Peptic ulcer; Treatment; Azithromycin; *H pylori*; Non-ulcer dyspepsia

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INTRODUCTION

H pylori is a common human pathogen that has been shown to be a major factor in peptic ulcer disease. It has also been linked to gastric adenocarcinoma and gastric lymphoma^[1].

A number of antimicrobial agents have been used in various regimens to eradicate *H pylori*. Clinical trials are regularly undertaken to search for simpler but equally effective regimens^[2-4]. Azithromycin, a new generation macrolide, has some special attributes that suggest it would be a promising compound to be used in regimens for *H pylori* eradication. It is acid-stable, has a long half-life and achieves remarkably high concentration in the gastric tissue^[3,5]. There have been several clinical trials of azithromycin for the therapy of *H pylori* infection^[3,4,6,7]. As the pharmacological properties of azithromycin make it possible to use shorter courses, the problem has been to define an optimal dose and duration of azithromycin treatment in the course of therapy^[3,4]. On the other hand, in developed countries, regimens of one week's duration are recommended for *H pylori* eradication^[1,2] but in developing countries 1-wk regimens failed to eradicate the microbe. A minimum of 10 to 14 d of treatment were needed for eradication of the microbe, even in the presence of a favorable sensitivity profile^[8-10]. The aim of this study was to assess the efficacy of azithromycin in a 1-wk regimen compared with a conventional 2-wk regimen in Iran, so we compared two quadruple regimens; bismuth subcitrate, omeprazole, azithromycin, and metronidazole, for 1-wk (B-OAzM) and bismuth subcitrate, omeprazole, amoxicillin and metronidazole, for 2-wk (B-OAM) in *H pylori* eradication. The safety and tolerability of the two drugs combinations were also evaluated and compared.

MATERIALS AND METHODS

Patients considered for the study included individuals 18-80 years old with upper GI symptoms that were referred to our gastroenterology clinic for upper endoscopy. Patients with *H pylori* infection were included in the study. Other inclusion criteria included indication for treatment as per the National Institutes of Health (NIH)

Table 1 Clinical and demographic data of patients in the treatment groups

Data	B-OAM-2 wk regimen n (%)	B-OAzM-1 wk regimen n (%)	P value ¹
ITT analysis	71	58	
Age: yr, Mean (SD)	48.3 (7.4)	46.7 (5.4)	0.17
Male	45 (63.3)	33 (56.8)	0.45
NSAID users	21 (29.5)	15 (25.8)	0.63
Cigarette smokers	11 (15.4)	8 (13.7)	0.78
Abdominal pain	47 (66.1)	31 (53.4)	0.14
Heartburn	14 (19.7)	18 (31)	0.13
Dyspepsia	57 (80.2)	40 (68.9)	0.13
PU	37 (52.1)	25 (43.1)	0.3
Non-ulcer dyspepsia	34 (47.8)	33 (56.8)	0.3
Loss to follow up	2	1	
Poor compliance	3	2	
Drop-outs	5	3	
PP analysis	66	55	

¹t; for means and χ^2 for proportions, ITT; intention to treat, PP; per protocol.

Consensus Conference including: peptic ulcer disease, history of peptic ulcer, chronic gastritis, gastric mucosa associated lymphoid tissue, or intestinal metaplasia^[1,7]. Between October 2003 and October 2004, a total of 129 patients were enrolled in the study. All patients gave written informed consent before entering the study and the protocol was reviewed and approved by the Semnan Gastrointestinal and Liver diseases Research Center. The criteria for exclusion were: intake of proton pump inhibitors, antibiotic or bismuth salts within 4 wk prior to the study, stomach surgery, known hypersensitivity to one of the study medications, patients with liver cirrhosis, renal failure or other serious severe concomitant illness, pregnant women, and patients who had previously undergone eradication therapy. These criteria were ascertained by taking a complete history, physical examination, and appropriate hematological and biochemical tests. The demographic and endoscopic data of these patients are reported in Table 1.

On initial endoscopy, *H pylori* infection was determined by rapid urease test and histological assessment. Rapid urease test was performed using two biopsy specimens; one from the antrum and the other from the corpus^[11]. Histological assessment of *H pylori* status was performed using one further biopsy specimen from the antrum; within 3 cm of the pylorus (hematoxylin-eosin and Giemsa stains)^[12]. The patients were considered to be infected if both the urease test and histology were positive on initial testing. The patients that satisfied the inclusion criteria were randomly assigned to one of the following regimens; one group received a regimen of bismuth subcitrate 240 mg, omeprazole 20 mg, azithromycin (Azithromycin TC®, Tehran shimi, Iran) 250 mg and metronidazole 500 mg, all bid, for 1-wk (B-OAzM) and the second group received a regimen of bismuth subcitrate 240 mg, omeprazole 20 mg, amoxicillin 1 g and metronidazole 500 mg, all bid, for 2-wk (B-OAM). Patients with duodenal or gastric ulcers continued on omeprazole (20 mg/d) for a total of 1 mo. Repeat examination was performed 1 wk and 4-8 wk after the cessation of therapy. Subjects recorded any side effects

and change in symptoms, and performed an exit interview and pill count to evaluate compliance and side effects. Hematological and biochemical analyses were performed at the last visit. *H pylori* infection was determined by urea breath test (UBT) at the second examination. All patients were instructed to discontinue all proton pump inhibitors, H2 blockers, and bismuth for at least 4 wk before UBT. Eradication was defined on the basis of a negative UBT (carbon 13-Isomax 2000 TM device); results under the 5 cut off were considered negative^[13].

Analysis of the two groups was conducted in the form of both per protocol (PP) and intention to treat (ITT). The ITT analysis for eradication was defined before the study to include all subjects who were randomized and received at least one dose of medication. The PP analysis for eradication included all subjects who took at least 80% of each study medication as prescribed.

The comparison of efficacy was evaluated using the χ^2 test. The analysis was performed using the SPSS 11.5 statistical package (SPSS, Chicago, IL).

RESULTS

The two groups were comparable in terms of common clinical variables that are summarized in Table 1. Three patients were lost to follow up (two from the B-OAM regimen and one subject from the B-OAzM regimen); also five patients discontinued the drugs because of severe side effects (three from the B-OAM regimen and two subject from the B-OAzM regimen). The remaining 121 patients completed the study as planned.

PP analysis: *H pylori* infection was eradicated in 43 of 55 patients in the B-OAzM group (78.18%, CI0.95: 64.98%-88.18%) and in 50 of 66 patients in the B-OAM group (75.75%, CI0.95: 63.63%-85.46%); the difference was not statistically significant ($\chi^2 = 0.1$, $P = 0.75$).

In patients with peptic ulcer, *H pylori* infection was eradicated in 19 of 25 patients in the B-OAzM group (76%, CI0.95: 54.87%-90.64%) and in 28 of 37 in the B-OAM group (75.67%, CI0.95: 58.8%-88.22%); the difference was not statistically significant ($\chi^2 < 0.001$, $P = 0.97$).

ITT analysis: *H pylori* infection was eradicated in 74.13% (CI0.95: 60.95%-84.74%) in the B-OAzM group and 70.42% (CI0.95: 58.4%-80.67%) in the B-OAM group; the difference was not statistically significant ($\chi^2 = 0.3$, $P = 0.58$).

Complications: the overall results for side effects are summarized in Table 2. Complications were noted in 14 patients in the B-OAzM group (25.45%) and 17 patients in the B-OAM group (25.75%) with no statistically significant differences between the groups ($\chi^2 < 0.001$, $P = 0.96$). The symptoms were mild and did not necessitate any additional treatment except in the five patients that discontinued the drugs for severe complications.

DISCUSSION

Although various regimens have been recommended for *H pylori* eradication, all of them include at least 2 antibiotics and one acid inhibitory drug^[1]. In western countries,

Table 2 Complications of drugs in *H pylori* treatment

Complication	B-OAM-2 wk regimen	B-OAzM-1 wk regimen	P value ²
<i>n</i>	69 ³	57 ³	
Diarrhea (%)	4 (5.7)	4 (7)	
Vomiting (%)	5 (7.2) ¹	4 (7) ¹	
Abdominal pain (%)	7 (10.1)	5 (8.7)	
Bad taste (%)	7 (10.1)	5 (8.7)	
Anal pain (%)	0	2 (3.5)	
Total (%)	23 (33.3%)	20 (35%)	0.83

¹Three patients of group "A" and two patients of group "B" excluded from the study for severe vomiting.; ² χ^2 ; ³ All patients except lost to follow up cases.

the most effective and usual regimens for preliminary treatment include; triple regimens for at least 1 wk, and use of clarithromycin as the antibiotic of choice against *H pylori*. Metronidazole, on the other hand, has largely been eliminated from first-line *H pylori* therapy because of its intolerability and high drug resistance^[14]. However, in developing countries, effective treatments for *H pylori* vary from those used in developed countries. For example in middle east countries it has been shown that one week regimens fail to eradicate the microbe and the course of treatment should be continued for at least 10-14 d to provide for eradication^[8-10]. Further, clarithromycin is not appropriate use because of its high price, drug resistance and unavailability^[15,16], and so metronidazole is a common and effective drug in *H pylori* treatment in this setting.

Although the prevalence of *H pylori* strains that are resistant to metronidazole varies from 46%-51% in Iran^[15-17], it has been shown that this drug in high doses (> 1 g) and in combination with other drugs remains effective against *H pylori*^[18-21]. Therefore, in Iran as also done in some Asian countries^[14,18] metronidazole is a very common drug used in *H pylori* eradication regimens^[22]. In Iran, the most common regimens for first-line treatment are 2-wk quadruple regimens that include; metronidazole, omeprazole, bismuth and tetracycline or amoxicillin^[22]. This conventional regimen introduced initially by Hosking, Deboer, Borody, and Laine as an effective regimen in *H pylori* treatment has confirmed efficiency of a 63%-93% eradication rate^[7,23-25]. On other hand, due to high rates of resistance to metronidazole, furazolidone was used instead of it, as first or particularly second line treatment^[26].

Although 2 wk regimens have been effective in *H pylori* eradication, some patients withdraw from their treatment because of the long duration and large number of tablets. Thus, in this study we showed that a quadruple regimen, where azithromycin replaced amoxicillin, the duration of the treatment can be decreased without any change in its effectiveness.

Azithromycin is a new macrolide related to clarithromycin with an effective role in *H pylori* eradication and it was reported that azithromycin has a synergic effect with esomeprazole, even in presence of drug resistance^[27]. In various studies the effectiveness of azithromycin has been evaluated in different doses from 500 mg to 3 g^[2,27-29], although in most studies this drug was used for 3 d (1.5 g

as in respiratory tract infection but at this dose it has less effect than clarithromycin^[3,5]. However, by increasing the total dose to 3 g, it has been shown that the effectiveness of azithromycin can be restored^[3,4,6,7].

This is the first study in which azithromycin was used at a prescribed amount of 3.5 g (250 mg bid for 7d) in the treatment of *H pylori* infection. Fortunately; the patients' tolerance was excellent. There were a few side effects based on biochemical tests, but most side effects were mild and disappeared with conservative therapy without the need to terminate the treatment.

Finally, in areas where clarithromycin cannot be used because of drug resistance or unavailability, azithromycin with a total dose of 3.5 g is an appropriate and safe drug for *H pylori* eradication regimen.

The effectiveness of furazolidone based *triple or quadruple regimens* has been evaluated in Iran^[15,22,25,26], but because of resistance to metronidazole, it seems possible that a combination of azithromycin and furazolidone instead of azithromycin and metronidazole will achieve more favorable eradication rates, although further evaluation is needed.

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Role of a probiotic (*Saccharomyces boulardii*) in management and prevention of diarrhoea

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of the side effects during treatment period.

CONCLUSION: *S. boulardii* significantly reduces the frequency and duration of acute diarrhoea. The consistency of stool also improves. The drug is well-tolerated.

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Key words: Acute watery diarrhoea; Probiotic; *Saccharomyces boulardii*; Frequency of episodes of diarrhoea; Weight gain

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Abstract

AIM: To assess the efficacy and safety of *Saccharomyces boulardii* (*S. boulardii*) in acute watery diarrhoea and its role in reducing the frequency of episodes of diarrhoea in subsequent two months.

METHODS: Children from 2 mo to 12 years of age, with acute diarrhoea were selected according to inclusion criteria and randomised in *S. boulardii* group (treated with ORS, nutritional support and *S. boulardii*, 250 mg bid) and in control group (treated with ORS and nutritional support only). Active treatment phase was 5 d and each child was followed for two months afterwards. Frequency and consistency of stools as well as safety of drug was assessed on every visit. A comparison of two groups was done in terms of number of diarrhoeal episode in subsequent two months.

RESULTS: There were fifty patients in each group. Baseline characteristics such as mean age and the average frequency of stools were comparable in *S. boulardii* and control group at the time of inclusion in the trial. By d 3 it reduced to 2.7 and 4.2 stools per d respectively and by d 6 it reduced to 1.6 (*S. boulardii* Group) and 3.3 (control group). The duration of diarrhoea was 3.6 d in *S. boulardii* group whereas it was 4.8 d in control group ($P = 0.001$). In the following two months, *S. boulardii* group had a significantly lower frequency of 0.54 episodes as compared to 1.08 episodes in control group. The drug was well accepted and tolerated. There were no reports

INTRODUCTION

Acute infectious diarrhoeal disease is a worldwide problem with over two million deaths each year. Diarrhoeal diseases are a leading cause of childhood morbidity and mortality and over 200 000 children die every year (600 deaths per day) in Pakistan. Repeated episodes of diarrhoea lead to under-nutrition. In Pakistan, every child gets, on average, 5-6 episodes of diarrhoea per year.

Last couple of decades has seen greater understanding of pathogenesis and simple methods of management of diarrhoea. Various modalities of interventions have been used in different parts of the world to improve the diarrhoeal mortality and morbidity which include oral rehydration salt (ORS), cereal based ORS, antibiotics, anti-diarrhoeals, antispasmodics and anti-emetics. Some of these modalities later proved to have variable harmful effects. These harmful effects include worsening of diarrhoea, increased duration of diarrhoea, adverse effects on gut motility leading to paralytic ileus. In addition, there are other systemic untoward effects^[1,2].

Gastrointestinal disease is often a consequence of a myriad of factors, which disturb the bowel's complex ecosystem. The concept of modulating bacterial activities, directed towards improving gut microbial function, has a long history. The use of yoghurt (as probiotic) in the treatment of diarrhoea has been known for a long time. It is now recognised that the most frequently used method of influencing the gut flora composition is that of probiot-

ics. A probiotic is a living micro organism administered to promote the health of the host by treating or preventing infections owing to strains of pathogens^[3]. Bio-therapeutic agents are defined as probiotics registered as drugs.

The modern view of probiotic therapy derives from the concept of a well functioning gut barrier and a normal balanced microbiota.

Numerous probiotic agents have been studied for the management of diarrhoeal disease. In particular, the prevention and management of acute viral diarrhoea, the treatment of recurrent *Clostridium difficile* diarrhoea, as well as the control of antibiotic-associated diarrhoea seem to be areas of significant potential benefit. A few agents, including *Lactobacillus GG*, *Lactobacillus reuteri*, and *Saccharomyces boulardii* (*S. boulardii*), seem to be promising agents for the amelioration of the course of acute diarrhoea in children when used therapeutically^[4]. Amongst these, all are bacteria except *S. boulardii*, which is yeast.

S. boulardii is a non-pathogenic yeast first isolated from lychee fruits in Indonesia and used first in France to treat diarrhoea, in the beginning of the 1950s. A lyophilised form is in clinical use in Europe, Asia, Africa, and Central and South America.

Preclinical and experimental studies of *S. boulardii* have demonstrated an anti-inflammatory, antimicrobial, enzymatic, metabolic and antitoxic activity. *S. boulardii* secretes a 54-KDa protease which has been shown to neutralize certain bacterial toxins; *S. boulardii* is also able to stimulate an immune response in the intestinal mucosa. It has a trophic effect by enhancing the metabolic function of the mucosa. *S. boulardii* releases polyamines, which are implicated in stimulating the enzymatic activity of the colonic mucosa^[3,5].

Based on our previous experience of use of *S. boulardii* in the treatment of diarrhoea, this study was undertaken to assess the efficacy of *S. boulardii* in the treatment of diarrhoea and reoccurrence of diarrhoea in subsequent two months.

MATERIALS AND METHODS

Patients

This randomised controlled clinical trial was carried out at Kharadar General Hospital, Karachi, catering to the needs of approximately one million population and situated in the middle and low-income community. An informed consent was obtained from parent/guardian of every child included in the trial. The children from 2 mo to 12 years of age presenting with acute watery diarrhoea of mild to moderate severity, fulfilling the inclusion criteria were included in this trial.

Children with severe inter-current illnesses, severe diarrhoea and dehydration requiring hospitalisation and intravenous therapy, presenting with temperature above 38.5°C, who were treated by any other anti-diarrhoeal/antibiotics in last 24 h as well as severely malnourished children were excluded from the trial. At inclusion, stool specimen was sent for bacterial culture and sensitivity as well as for Rota virus detection.

The study population of 100 children was randomised into two groups. In *S. boulardii* group, patients were

Table 1 Baseline characteristics

Patient characteristics	<i>S. Boulardii</i> (n = 50)	Control (n = 50)	P value
Age (mo)			
Mean	18.3	26.01	0.08
SD	20.33	23.37	
Weight (kg)			
Mean	8.2	9.15	0.224
SD	3.58	4.13	
Sex			
Female	50%	50%	1
Male	50%	50%	
Stool culture/Sensitivity	26%		
Bacteria isolated	74%	12%	0.125
Not isolated		88%	
Rota virus			
Positive	16%	20%	0.795
Negative	84%	80%	

managed by WHO-CDD protocol^[6] plus *S. boulardii* (250 mg bid) administered orally diluted in water or other semi-solid food. The product was manufactured by Hilton Pharma (Pvt) Ltd. under the license of Biocodex, France. In the control group patients were managed by WHO-CDD protocol only. The active treatment period was 5 d. Treatment of the subsequent episodes of diarrhoea was at the discretion of the treating physician.

All study participants were examined on d 0 (inclusion day), and followed up on d 3 and d 6 during active treatment phase and every month for two months thereafter for observation.

The first visit data collection included date of onset of diarrhoea, previous treatment (where applicable), weight of child, number and consistency of stools, vomiting, body temperature, sign of dehydration and any other data by clinical examination. The second visit information variables included date of stoppage of diarrhoea in case of inter-current recovery, weight of child, daily record of frequency and consistency of stools, tolerance and acceptability of treatment. Similarly on third visit, date of stoppage of diarrhoea in case of inter-current recovery, weight of child, daily record of frequency and consistency of stools, tolerance and acceptability of treatment were recorded in the study record forms.

A monthly observational follow-up data for two months included weight of child at monthly interval and any new episodes of diarrhoea in both the groups.

Statistical analysis

For statistical analysis, *t*-test was applied to measure the variation in means. $P < 0.05$ was taken as significant.

RESULTS

One hundred patients were analysed in the study, fifty patients in each group. Patient baseline characteristics in control and study groups were comparable (Table 1). Bacteria were isolated in 26% and 12% of the *S. boulardii* and control groups respectively while Rota virus detection

Table 2 Mean numbers of stools and duration of diarrhoea

	<i>S. boulardii</i>	Control	P value
Mean number of stools reported on d 0	9.5	8.8	0.37
Mean number of stools reported on d 3	2.8	4.4	0.01
Mean number of stools reported on d 6	1.6	3.3	0.001
Duration of diarrhoea (d)	3.6	4.8	0.001

Table 3 Number of episodes of diarrhoea

	<i>S. boulardii</i>	Control	P value
No. of episodes at 1 mo	0.2	0.64	0.001
No. of episodes at 2 mo	0.32	0.56	0.04

Table 4 Weight gain

	Group	% of mean weight gain	P value
% increase in wt. at 1 mo	<i>S. boulardii</i>	4.4	0.902
	Control	4.2	
% increase in wt. at 2 mo	<i>S. boulardii</i>	9.9	0.067
	Control	6.2	

revealed 16% and 20% positive for test and control group respectively.

On d 3 and d 6, there was a significant reduction in reported number of stools in *S. boulardii* group as compared to the control group. The mean duration of diarrhoea was 3.5 d in *S. boulardii* group and 4.8 d in the control group ($P = 0.001$) (Table 2).

Follow-up for the next two months also revealed interesting results. Mean numbers of episodes of diarrhoea by the end of two months, were 0.56 in control group as compared to 0.32 in *S. boulardii* group, which is almost half of that of control group (Table 3).

S. boulardii was well accepted and tolerated by the children and there were no reports of any side effects during the study period.

Table 4 also shows mean weight gain in both groups at mo 1 and 2. Although the difference of gain between the two groups has not reached statistical significance, the percentage of average increase in the experimental group was 9.9% as compared to 6.2% in the control group.

DISCUSSION

There are over 10^{9-12} bacteria per gram of faeces and about 400 different species, more than 10 times the human cells^[7]. The intestinal flora is intimately associated with the organ which contains it and with which it forms an ecosystem. Equilibrium within this ecosystem is essential to good health of the individual. It influences the structure, motility, physical and chemical conditions of intestinal tract, metabolic and enzyme activity of

mucosa and establishment and maturation of immune system. Finally and above all, the intestinal microbial flora forms a true resistance to colonisation of digestive tract by pathogenic microorganisms. Disruption of intestinal ecosystem occurs in many pathological situations such as infectious diarrhoea or diarrhoea and colitis linked to antibiotic treatment.

Despite awareness about preventive aspects of diarrhoea it remains one of the leading causes of morbidity and mortality in children, because of lack of clean water supply and sanitation.

Search for newer, less harmful agent is continued. Biological agents ("biotherapeutic agents" or "probiotics") have been used to treat a variety of infections, most notably infections of mucosal surfaces such as the gut and vagina. These biotherapeutic agents include certain bacteria and the yeast *S. boulardii*. Given orally, *S. boulardii* seem to be promising agents for the amelioration of the course of acute diarrhoea in children^[8].

The current study was based on our previous clinical observation, which revealed that children treated with *S. boulardii* had a decreased number of episodes of diarrhoea in following months.

This study verified our previous observation, as there was a 50% reduction in the number of episodes of diarrhoea in the treatment group as compared to control group (Table 2).

This study also showed a significant improvement in frequency and consistency of stool and reduction in duration of illness in patients who were given *S. boulardii* along with WHO-CDD protocol.

Several studies of *S. boulardii* have been done in children and adults in the treatment of acute diarrhoea^[9]. The results of our studies are consistent with some of these studies. However, the current study is the first one to observe the reduction in number of episodes of diarrhoea in the post-treatment follow-up period of two months. Stimulation of local immunity, as demonstrated by the increase of IgA, together with the enhancement of the trophic activity of the mucosa (through the release of polyamines) by *S. boulardii* may, at least in part, explain the long term effect of the yeast.

A meticulous follow-up of the patient resulted in very good compliance. No side effects were observed during the active treatment period with the use of *S. boulardii*. McFarland *et al* also highlight the safety profile in their review on *S. boulardii*^[10].

CONCLUSION

Based on our experience of this trial we conclude that *S. boulardii* is a useful and welcome addition to the treatment of acute diarrhoea in children. *S. boulardii* reduces the frequency of stool, and duration of illness. It also reduces the number of episodes of diarrhoea by 50% in the subsequent period of two months.

Though this is the first study of its kind in Pakistan, we are of the opinion that multicenter double blind placebo controlled trials need to be conducted to confirm our observations. Investigators have been using probiotics as

prophylaxis in childhood infection whereby it has been shown to reduce the rate of infection^[7,11].

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Intraoperative endoscopy in obstructive hypopharyngeal carcinoma

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Abstract

AIM: To demonstrate the necessity of intraoperative endoscopy in the diagnosis of secondary primary tumors of the upper digestive tract in patients with obstructive hypopharyngeal carcinoma.

METHODS: Thirty-one patients with hypopharyngeal squamous cell carcinoma had been operated, with radical intent, at our Institution in the period between 1978 and 2004. Due to obstructive tumor mass, in 7 (22.6%) patients, preoperative endoscopic evaluation of the esophagus and stomach could not be performed. In those patients, intraoperative endoscopy, made through an incision in the cervical esophagus, was standard diagnostic method for examination of the esophagus and stomach.

RESULTS: We found synchronous foregut carcinomas in 3 patients (9.7%). In two patients, synchronous carcinomas had been detected during preoperative endoscopic evaluation, and in one (with obstructive carcinoma) using intraoperative endoscopy. In this case, preoperative barium swallow and CT scan did not reveal the existence of second primary tumor within esophagus, despite the fact that small, but T2 carcinoma, was present.

CONCLUSION: It is reasonable to use intraoperative endoscopy as a selective screening test in patients with

obstructive hypopharyngeal carcinoma.

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Key words: Hypopharynx; Neoplasms; Squamous cell; Endoscopy; Multiple primary; Synchronous tumors

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INTRODUCTION

The rate of synchronous primary cancers in the upper aerodigestive tract in the reported literature varies widely depending on the population surveyed and the thoroughness of the methods used to evaluate these patients^[1]. In 25 studies published over 25 years, the average rate of synchronous primary upper aerodigestive cancers was 4%, ranging from 1.5% to 18%^[2]. The relative risk of developing esophageal cancer in patients with head and neck cancer has been reported as being 3-20 times greater than that of control subjects from the general population^[3-5]. The phenomenon of neoplastic multicentricity could affect the therapeutic approach, and cause local treatment failure.

The aim of this study was to demonstrate the necessity of intraoperative endoscopy in the diagnosis of secondary primary tumors in upper digestive tract in the patients with obstructive carcinoma of the hypopharynx.

MATERIALS AND METHODS

In the period between January 1st, 1978 and January 1st, 2004, 31 patients with hypopharyngeal squamocellular carcinoma had been operated at the Department of Esophagogastric Surgery, First University Surgical Hospital, Clinical Center of Serbia. In most patients a complete preoperative work-up was performed. Tumor resectability was assessed by means of chest X-ray, barium swallow, esophagoscopy (flexible and rigid), tracheobronchoscopy, ENT evaluation, thoracic and neck CT scan and ultrasonography. In patients with obstructive

lesions, where preoperative esophagogastrosopic evaluation was not feasible, intraoperative endoscopy (with lugol staining method) through cervical esophagotomy was performed (Figure 1). Intraoperative endoscopy was performed in standard manner with diagnostic fiberoptic endoscope. Instrument was introduced in the esophagus through incision in the esophageal wall, made below lower border of the tumor. When tumor involved cervical esophagus and extended to the thoracic inlet, procedure was not feasible.

For the diagnosis of multiple separate (synchronous) primary carcinomas we followed standard criteria^[6]: (1) neoplasms must be clearly malignant as determined by histological evaluation; (2) each neoplasm must be geographically separate and distinct. The lesions should be separated by normal-appearing mucosa. If a second neoplasm is continuous to the initial primary tumor, or is separated by mucosa with intraepithelial neoplastic change, two lesions should be considered as confluent growth rather than multicentric carcinomas; (3) the possibility that the secondary neoplasm represents a metastasis should be excluded. The observation that the invasive carcinoma arises from an overlying epithelium, which demonstrates a transition from carcinoma *in situ* to invasive carcinoma is helpful, and when the separate foci have significant difference in histology, the diagnosis of separate primary cancer is appropriate.

RESULTS

Mean age of the patients in our study was 53.5 years (range 36-72 years). The male to female ratio was 1:2.1. The most common complaint was dysphagia (83.9%), indicating an advanced disease. We had no experience with stage I and II carcinoma. Stage III and IV accounted for 23 (74.2 %) and 8 (25.8%) respectively. Due to obstructive tumor mass, in 7 (22.6%) patients, preoperative contrast radiography was not conclusive, and preoperative endoscopic evaluation could not be performed. Obstructive tumors were predominantly localized in postcricoid region, and mostly infiltrating esophageal ostium. In those patients intraoperative endoscopy, made through incision on the cervical esophagus (below hypopharyngeal tumor), was standard diagnostic method for examination of the esophagus and stomach.

In our series, we found synchronous foregut carcinomas in 3 patients (9.7%), two of them with synchronous carcinoma of the thoracic esophagus, and one with stomach carcinoma. In two patients, synchronous carcinomas had been detected during preoperative endoscopic evaluation, and in one patient (with obstructive carcinoma) using intraoperative endoscopy. In this patient, preoperative barium swallow and CT scan did not detect existence of second primary tumor within esophagus despite the fact that small, but T2 carcinoma, was present.

DISCUSSION

With regards to risk factors for head and neck, or esophageal carcinomas, genetic factors and environmental factors, such as smoking and alcohol, have been reported



Figure 1 Intraoperative endoscopic evaluation through cervical esophagotomy, made just below the hypopharyngeal carcinoma.

to be important^[7,8]. These data suggest the concept of "field carcinogenesis"^[6]. According to Martins^[9] and Kumagai^[10] more than 70% of patients with synchronous hypopharyngeal carcinoma have second malignancy in the esophagus, but second gastric malignancy could not be detected. Kodama *et al*^[11] also reported high prevalence of synchronous carcinomas of the pharynx and esophagus. Gluckman *et al*^[12] recommended the use of panendoscopy in the evaluation of all head and neck cancers. Likewise, McGuirt^[13], Leipzig^[14], and Shapshay^[15] also recommended panendoscopy due to frequent association of head and neck, esophageal and lung carcinomas.

One of the most difficult problems we confront clinically, in the preoperative examination, is the subgroup of patients where esophageal and gastric fiberscopy (GIF) could not be performed due to obstructive hypopharyngeal mass. Without knowing the existence of multiple lesions, we cannot decide the proper therapeutic (surgical) plan. Martins *et al*^[9] reported that esophagoscopy and esophagography was attempted in 97% of patients in their series, but these examinations failed to evaluate the entire esophagus in 46% patients because of severe obstruction. In addition, very few patients (5 out of 36) underwent lugol staining during esophagoscopy. Although all patients were appropriately studied preoperatively, in only two cases multiple tumors were diagnosed before surgery. The remaining multiple synchronous carcinomas were obviously missed. Second primary tumor was, as a rule, located distal to the main obstructing carcinoma, preventing adequate total esophageal examination. Akiyama^[16] cited intraoperative esophagoscopy as an important step in such patients. However, this procedure is not possible when tumors widely infiltrate cervical esophagus. Intraoperative endoscopy made through incision below the tumor, could be performed in most patients with obstructive carcinoma of postcricoid region, even when tumors infiltrate the esophageal ostium.

To justify the selective use of intraoperative endoscopy as a diagnostic tool for patients with obstructive hypopharyngeal carcinoma, a number of issues need to be considered. The procedure should significantly improve the diagnosis of synchronous primary tumors when compared with non-invasive radiologic investigations, including barium swallow (BaSw) and chest computed tomography (CT) or magnetic resonance imaging (MRI). Symptom directed studies are not feasible because patients already have severe dysphagia due to obstructive proximal



Figure 2 Synchronous invasive carcinomas of the hypopharynx and thoracic esophagus.

carcinoma. In addition, second primary tumor could be of early stage and does not cause subjective symptoms even in absence of proximal obstruction. Kohmura *et al*^[6] support the view that in patients with obstructive hypopharyngeal carcinomas MRI should be performed. According to this data, esophageal mass lesions or hypertrophic mucosa detected by MRI require esophageal blunt dissection, due to possibility of multiple primary malignancies. In addition, Kohmura *et al*^[6] proposed that there is little chance of multiple malignancies in the esophagus if there are no abnormalities by MRI. They also pointed out that endoscopic evaluation is favorable whenever is feasible. However, many authors agree that the role of MRI in the examination of gastrointestinal tract, apart from the liver, is limited^[17,18]. CT has shown similar limitations in the diagnosis and staging of esophagogastric tumors with accuracy rate of only 60% or less^[19,20]. Many others^[21-23] agree that endoscopic ultrasonography (EUS) has clearly showed superior accuracy for T and N staging of esophageal and gastric carcinoma as compared with CT, but has the same limitations as endoscopic examination in the patients with obstructive tumors. Miniprobe sonography showed favourable results in the diagnosis and staging of esophageal tumors^[18], but unfortunately, in most institutions is not available in routine clinical practice.

The second issue to be addressed is whether the diagnosis of a second primary tumor would change the primary treatment approach for the individual patient. Panosetti *et al*^[24] in large series of patients, demonstrated that the discovery of a synchronous second primary tumor altered the treatment approach in 50% of patients. Many reports favour free jejunal interposition as a reconstructive method rather than gastric transposition^[25-27]. These approaches may leave behind a premalignant or malignant lesion in the esophagus. One of the strongest arguments for total pharyngolaryngoesophagectomy and gastric transposition is the presence, or possibility of synchronous or metachronous primary in the esophagus. But then, stomach should be completely evaluated by endoscopy and x-ray before surgery, due to possibility of presence of the malignant tumor within stomach. Since the stomach is commonly used for reconstruction of the digestive tract after esophagectomy, another substitute must be considered for patients with synchronous gastric cancer.

The third issue to consider is the prognosis of patients with synchronous primary tumors, and impact of detection

on overall survival. Advances in therapeutic methods have significantly improved local control rates. Still, many patients are developing distant metastases. Panosetti *et al*^[24] reviewed the impact of survival of patients with synchronous versus metachronous second primary tumors. In a large series, these authors demonstrated that patients who were initially seen with synchronous primary tumors had 5-year survival rates of 18% vs 55% for those with metachronous tumors. These suggest that the overall survival for patients with detectable synchronous primary is quite poor. Martins *et al*^[9] showed that more than 80% of second synchronous primaries were invasive carcinomas. In contrary, Kumagai^[10] and Kohmura^[6] found that most of the esophageal carcinomas accompanying advanced hypopharyngeal carcinomas were of early stage and that surgical excision could positively influence the prognosis. Thus, with the possibility of multiple intraesophageal cancer, endoscopic screening of the esophagus with lugol dye method in patients with head and neck cancer is necessary before treatment^[13,28,29]. In our series, single synchronous primary was relatively small, but invasive (pT2) tumor of the thoracic esophagus, was visible without lugol staining (Figure 2).

Considering low incidence of obstructive hypopharyngeal carcinomas and high sensitivity of intraoperative endoscopy in detection of second primary, there is no need to consider whether this diagnostic procedure is cost-effective or not. More important is that there are no complications related to the procedure. One of the things that might be of concern is potential contamination of the operating field during the procedure. In our experience there were no local infective complications associated with the procedure. Therefore, benefit of the procedure exceeds potential risk of local contamination.

In summary, it is reasonable to use intraoperative endoscopy as selective screening test in patients with obstructive hypopharyngeal carcinoma.

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Prognostic value of additional pathological variables for long-term survival after curative resection of rectal cancer

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Abstract

AIM: To evaluate the prognostic value of some pathological variables in rectal cancer survival.

METHODS: 247 patients who underwent curative resection of rectal cancer were included in the study. The influence on survival of five pathological variables (histopathological tumor type, histopathological tumor grade differentiation, blood vessel invasion, perineural invasion and lymphatic invasion) was assessed using statistical analyses.

RESULTS: Overall 5-year survival was 71.2%. Univariate analysis of all tested variables showed an effect on survival but only the effect of lymphatic invasion was statistically significant. At stages three and four it had a negative effect on survival ($P = 0.0212$). Lymphatic invasion also significantly affected cancer related survival in multivariate analysis at stages three and four. At lower stages (stage 0, stage 1 and stage 2) multivariate analysis showed a negative effect of perineural invasion on cancer related survival.

CONCLUSION: Patients with lymphatic and perineural invasion have a higher risk for rectal cancer related death after curative resection. Examination of these variables should be an important step in detecting patients with a poorer prognosis.

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Key words: Rectum; Cancer; Prognosis; Survival; Lymphatic invasion; Perineural invasion

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INTRODUCTION

Rectal cancer is often a curable disease and survival is directly correlated with the stage of the tumor, assessed by indicating the depth of penetration of the tumor into the bowel wall (T stage), the extent of lymph node involvement (N stage), and the presence of distant metastases (M stage). Besides the degree of penetration and presence of nodal involvement or distant metastasis, many other clinical, histological and biomolecular variables have been evaluated in the prognosis of patients with rectal cancer, although most have not been prospectively validated^[1-3]. Among those, pathological factors are especially important as they may help to sub stratify tumors of same stage into different risk categories^[4].

In Slovenia as in most countries, patients with radically resected rectal cancer with negative lymph nodes do not receive adjuvant systemic therapy, since significant survival benefits have not yet been proven^[5]. One third of those patients, however, will develop local or distant metastases^[6]. Identifying such patients would be very important because they could benefit from adjuvant systemic treatment.

The aim of our study was to retrospectively examine five histopathological prognostic factors which are routinely assessed and which may help to identify patients who could be included in an adjuvant chemotherapy program.

Histopathological tumor type (H)

The majority of colorectal cancers are adenocarcinomas. Some subtypes, like mucinous carcinoma (defined in the recent TNM classification as tumors with mucinous areas comprising more than 50% of tumor), signet ring cell carcinoma, and undifferentiated carcinoma, have a worse prognosis than adenocarcinoma.

Degree of differentiation (G)

In some multivariate studies, the microscopic grade is predictive of survival; a higher grade is known to be associated with a significantly increased risk of an adverse outcome.

Blood vessel invasion (BVI)

Blood vessel (venous) invasion by tumor has been demonstrated to be a stage-independent adverse

prognostic factor by many analyses^[7-9]. However, some studies identifying venous invasion as an adverse factor on univariate analysis have failed to confirm its independent impact on prognosis in multivariate breakdowns^[12].

Lymphatic invasion (LI)

Similar disparate results have also been reported for lymphatic invasion^[10-11]. Therefore, data from existing studies are difficult to interpret. Nevertheless, the importance of venous and lymphatic invasion by the tumor is strongly suggested and largely confirmed by the literature^[4].

Perineural invasion (PNI)

PNI may influence prognosis after resection of rectal cancer. An abundant extramural autonomic nerve network is an anatomical feature of the rectum. The evidence for the importance of perineural invasion is weaker than for blood vessel or lymphatic invasion but it has been shown in some series to be a predictor of both local failure and worse survival^[13-15].

MATERIALS AND METHODS

Since 1998, data from all rectal cancer patients in our institution have been simultaneously registered in specially designed protocols, which contain preoperative, operative and postoperative parts and detailed pathological report.

The stage of the rectal cancer was defined according to the recent TNM classification^[16].

Blood vessel invasion was considered positive if the following criteria were fulfilled: tumor cells at endothelial surface, tumor cell thrombi inside the lumen of the vessel or destruction of the vascular wall by tumor. Lymphatic invasion was defined as positive when tumor cells were found within endothelium-lined space devoid of mural smooth muscle or elastic fibers. Perineural invasion was considered positive when tumor cells were detected along, or around, a nerve within the perineural space.

Data from all patients who were operated on between January 1998 and December 2003 were analyzed. Over this period 512 patients with rectal carcinoma underwent surgery, but only 247 patients were eligible for the study, which included patients with elective radical resection of a solitary rectal tumor who survived the first month after the operation.

All patients were followed up on regular basis: at 3 mo intervals during the first 2 years, at 6 mo intervals until 5th year and then yearly. Clinical examination, tumor markers (CEA and CA19-9) and ultrasound were performed at each appointment. Chest X-ray was performed every 6 mo during first 2 years and then yearly. Colonoscopy was performed yearly after the operation. Follow up was updated on March 1st 2005.

We analyzed the following pathological variables: Histopathological tumor type, tumor grade or differentiation (well, moderately well, poorly differentiated and undifferentiated), blood vessel invasion, lymphatic and perineural invasion (present or absent).

Mortality data was acquired from the Slovenian cancer

Table 1 Basic demographic analysis

Age (yr)	65.8 (range 34-90) n (%)
Sex	
Men	138 (56)
Women	109 (44)
Follow up (d)	Median 1055 (range 31-2591)

Table 2 Distribution of patients according to their stage of disease

UICC	n = 247	%
0	2	1
1	54	22
2	116	47
3	72	29
4	3	1

register and cause was verified for each deceased patient.

The research received approval from our ethics committee.

Survival was calculated using the Kaplan-Meier method and compared by log-rank test. Multivariate analysis was performed using Cox's regression model. All analyses were performed using statistical software SPSS for Windows 10.0.

RESULTS

The main operative procedures for rectal cancer in our institution are radical low anterior resection and abdominoperineal resection. Both procedures are executed according to principles of total mesorectal excision.

All patients were postoperatively managed by oncologists. The majority of patients with stage 3 and 4 disease received postoperative chemotherapy if there were no general contraindications.

The mean follow up time was 1055 days. The basic demographic analysis is shown in Table 1.

Most of the patients had stage 2 disease (47%), 29% had stage 3 and 22% stage 1. Only one percent of patients were at stage 0 and stage 4, as shown in Table 2.

5-year cancer related survival was calculated using Kaplan-Meier method and was 71.4% for all stages.

For stage 1, survival was 91%, for stage 2 74%, for stage 3 51% and for stage 4 33% (Figure 1).

For further analysis patients were divided into two groups according to stage. One group consisted of patients with stage zero, one and two tumors, and the second group consisted of patients with tumor stage three and four (Table 3).

The histopathological type of tumor and the degree of differentiation affected survival in lower and higher stages but this difference was not statistically significant.

Vascular and perineural invasion also showed no effect on 5-year survival in lower and higher stages.

Lymphatic invasion showed no effect on survival in lower stages but in higher stages, positive lymphatic invasion had a statistically significant negative effect on survival (log rank, $P = 0.0212$) (Figure 2).

Table 3 Univariate survival analysis of pathological variables according to stage

Variable	All Cases		LOWER STAGES (Stages 0, 1 and 2)		HIGHER STAGES (Stages 3 and 4)	
	<i>n</i>	%	5-yr cancer related survival (%)	<i>P</i> (log-rank test)	5-yr cancer related survival (%)	<i>P</i> (log-rank test)
Histological type	<i>n</i> = 246					
Adenocarcinoma	214	87	79	0.4056	42	0.5016
Mucinous carcinoma	23	9	83		74	
Other types	9	4	/		66	
Differentiation	<i>n</i> = 235					
G1	70	30	77	0.1312	43	0.5792
G2	141	60	87		63	
G3	21	8.9	51		42	
G4	3	0.1	/		/	
Blood vessel invasion	<i>n</i> = 226					
Positive	28	12	71	0.5920	46	0.2009
Negative	195	88	77		54	
Perineural invasion	<i>n</i> = 224					
Positive	31	14	74	0.1143	31	0.4247
Negative	193	86	78		53	
Lymphatic invasion	<i>n</i> = 218					
Positive	30	14	85	0.9083	27	0.0212
Negative	188	86	76		68	

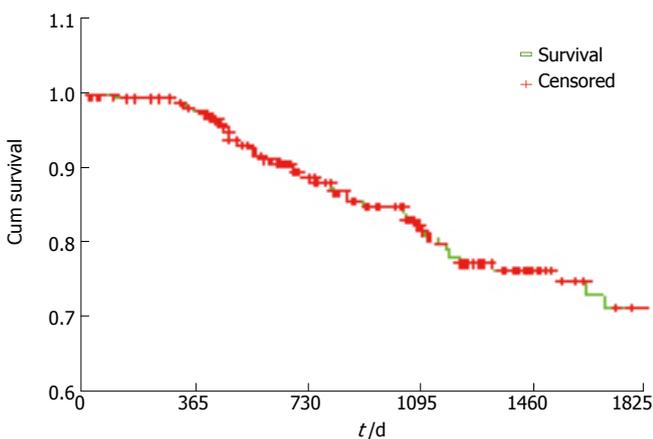


Figure 1 Cumulative 5-yr cancer related survival of all radically operated rectal cancer patients calculated with Kaplan-Meier method.

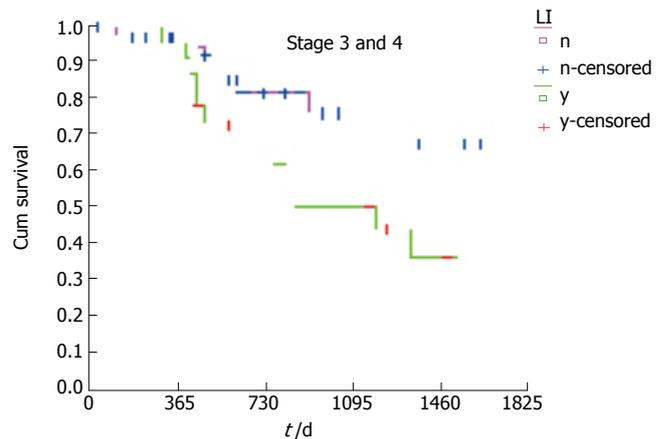


Figure 2 5-yr survival of radically operated rectal cancer (stage three and four) related to the presence or absence of lymphatic invasion. The difference is statistically significant ($P < 0.005$). Kaplan-Meier method.

Multivariate analysis

Using Cox regression model on all previous variables we obtained different results for the different stages. Examining patients with higher stages we again confirmed that lymphatic invasion is an important prognostic variable. In multivariate analysis, lymphatic invasion clearly emerged as a strong negative prognostic factor ($P = 0.022$).

On the other hand, when patients with lower stages were considered, another variable, perineural invasion, seemed to have the greatest effect on survival ($P = 0.036$).

DISCUSSION

Rectal cancer is one of the leading causes of cancer-related deaths in the developed world^[17].

Slovenia has one of the highest incidences of rectal cancer (men: 23.7 per 100 000, women 14.9 per 100 000). In 2001 there were 383 new cases of rectal cancer detected

and 128 cancer-related deaths^[18].

We know that more than two-thirds of patients with newly detected rectal cancer will undergo curative primary tumor resection and more than half will eventually die from the disease following surgery^[19]. Survival is clearly dependent on rectal cancer stage but there are many other variables that could help us in predicting outcome. During recent years there were many studies and methods which focused on identification of factors that could influence postoperative survival after curative rectal cancer resection. These include serial sectioning, immunohistochemistry, and polymerase chain reaction assays. Examining all regional lymph nodes with these methods would be preferred, but is expensive and time consuming and therefore not feasible in daily practice.

Sentinel node-mapping offers a potential solution. It has been introduced in colorectal cancer to improve staging by facilitating occult tumor cell assessment in lymph nodes

that are most likely to be tumor-positive^[20]. However, there is a large variation in identification rates and false-negative rates usually ascribed to the learning curve effect, differences in technique and tumor stage.

Compared to the methods described above, pathological variables, as assessed in this work, may have some advantages. They are not expensive and do not need any special resources.

In our study, lymphatic and perineural invasion were pathological variables that were significantly associated with poorer outcomes in high and low stage groups, respectively. The importance of lymphatic invasion was already recognized in the late 1980's. Minsky found that it was an independent prognostic factor by proportional hazard analysis^[21]. The importance of lymphatic invasion was confirmed in some later studies but also denied in others^[22]. In our work it had a clearly negative effect on survival in a univariate analysis. In a multivariate analysis, the negative effect was seen only in patients with node positive rectal cancer. We assume that positive lymph nodes facilitate lymphatic invasion that could precipitate further spreading of the disease.

According to available data, perineural invasion may influence the prognosis after resection of rectal cancer^[13]. In our work perineural invasion was associated with shorter survival in patients with lower stages of tumors. It is possible that the influence of positive perineural invasion in higher stages is masked by the importance of other parameters, and thus its role is clearly seen only in lower stages.

We must, however, mention some limitations of our study. First, although we report a 5-year overall survival, our median follow-up was 1055 d. However, we have used the Kaplan-Meier method to estimate 5-year survival. Second, we performed a retrospective study, and therefore our conclusions are limited by the bias inherent in an analysis of this nature.

In conclusion, additional pathological variables deserve more research to validate whether they will be helpful in predicting outcomes in rectal cancer patients. They are not expensive, are performed routinely and do not need any special resources. They could help with the identification of patients who would be predicted to have a poorer prognosis after curative resection of rectal cancer, who accordingly might benefit from adjuvant oncological treatment.

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Time trends of incidence of digestive system cancers in Changle of China during 1988-2002

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Abstract

AIM: To analyze the incidence of digestive system cancer in Changle of China over a 15-year period.

METHODS: The datasets were presented as time-series of China-standardized annual incidence during 1988-2002. Linear regression model was used to analyze the incidence of stomach, liver, esophagus and colorectal cancers.

RESULTS: Linear regression models for the time-series of stomach and esophagus cancer incidences for both men and women were statistically significant ($P < 0.05$); Regression models for liver cancer and for colorectal cancer were statistically significant for men ($P < 0.05$).

CONCLUSION: The incidence rates of stomach and esophagus cancers for both men and women had down tendencies. For men, liver cancer had a down trend of the incidence and colorectal cancer had an upward trend of the incidence rate.

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Key words: Stomach; Esophagus; Liver; Colorectum; Cancer; Incidence; Pattern; China

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INTRODUCTION

Cancer is the first cause of death in Fujian Province of

China, and the proportion of cancer-related deaths is the highest. The mortalities of malignant digestive system tumors in Changle during 1988-1991 were the highest in Fujian Province and the incidence (per 100 000 persons) of stomach, liver, esophagus and colorectal cancers in Changle is 104.3%, 37.3%, 25.6% and 7.4%, respectively^[1]. Since 1988, efforts have been made in prevention and treatment of tumors^[2]. However, the patterns and incidence of malignant digestive system tumors in Changle are not available. Monitoring and studying the incidence of malignant tumors can provide important information for prevention and control of tumors. Therefore, the aims of the present study were to examine the pattern and time trends of stomach, liver, esophagus and colorectal cancers from 1988-2002 in Changle.

MATERIALS AND METHODS

Cancer incidence data

Annual stomach, liver, esophagus and colorectal cancer cases were provided by the Tumor Registration Office of Changle. Data of the age- and sex-specific population in Changle were provided by the local police station. The incidence of these tumors was standardized with the age- and sex-specific population obtained from the National Population Overall Survey in 1983. The datasets were presented as time-series of China standardized annual incidence during 1988-2002.

Statistical analysis

Linear regression model was used to analyze the incidence of stomach, liver, esophagus and colorectal cancer. The SAS software package was used for all analyses^[3].

RESULTS

Standard incidence of digestive system cancer in men and women of Changle is shown in Table 1. The incidence of stomach, liver and esophagus cancer in men decreased with fluctuation while that of colorectal cancer increased slowly from 1988 to 2002 (Figures 1A and 1B). On average, the incidence of stomach, liver and esophagus cancer in males decreased 3.44%, 3.16% and 6.07%, respectively. The incidence of these tumors except for stomach cancer in females was not significantly different (Figure 1C). The incidence of stomach, liver and esophagus cancer in women decreased 2.21%, 1.20% and 2.50%, respectively. The incidence of colorectal cancer in men increased 5.51% from 1988 to 2002.

Table 1 Standard incidence of digestive system cancer per 100 000 persons in 1988-2002 in Changle (%)

Yr	Stomach cancer		liver cancer		Esophagus cancer		Colorectal cancer	
	Male	Female	Male	Female	Male	Female	Male	Female
1988	106.63	28.54	32.62	10.48	20.22	6.36	3.1	6.3
1989	114.41	36.55	35.09	9.87	24.96	8.18	4.96	4.69
1990	109.09	29.59	51.86	10.76	28.81	12.48	5.93	4.56
1991	103.21	34.52	38.62	12.78	30.76	9.95	4.89	1.18
1992	100.81	26.1	32.47	7.66	25.52	7.45	2.44	4.11
1993	111.65	27.35	36.25	14.36	27.22	7.91	2.82	2.86
1994	98.73	24.9	44.54	11.09	22.38	7.92	4.04	2.16
1995	80.95	20.9	32.71	9.87	18.57	8.13	5.58	5.57
1996	85.42	19.52	32.87	7.63	18.7	4	6.24	4.43
1997	86.4	26.56	27.86	7.81	14.47	2.77	9.6	4.68
1998	81.81	21.82	35.48	16.29	12.96	4.07	5.86	5.33
1999	75.84	20.71	32.76	9.2	12.54	3.59	6.76	3.86
2000	83.96	25.63	32.81	10.99	15.15	2.63	5.39	3.82
2001	74.28	27.71	33.12	5.17	11.15	4.29	9.12	5.38
2002	65.33	20.88	20.81	8.85	8.41	4.46	6.57	8.03

Table 2 Incidence of four tumors both in men and women in Changle from 1988 to 2002 (%)

Disease	Men			Women		
	Regression coefficient	R ²	P	Regression coefficient	R ²	P
Stomach cancer	3.16	0.851	< 0.001	0.72	0.415	0.009
Liver cancer	0.83	0.286	0.04	0.16	0.061	0.374
Esophagus cancer	1.33	0.736	< 0.001	0.48	0.567	0.001
Colorectal cancer	0.29	0.391	0.013	0.12	0.1	0.251

The incidence of digestive system tumors both in men and in women was analyzed by linear regression models (Table 2). The regression coefficients for both men and women, except for colorectal cancer, were negative. The incidence of stomach and esophagus cancer in both men and women decreased ($P < 0.05$). The incidence of liver cancer was decreased in men ($P < 0.05$) but not in women. The incidence of colorectal cancer increased in men ($P < 0.05$) but not in women.

DISCUSSION

This report presents the time-series analysis of standardized annual incidence of digestive system cancers in Changle City of China from 1988 to 2002. The incidence of stomach and esophagus cancers in men and women decreased 3.44% and 2.21%, respectively. The incidence of liver cancer decreased 3.16% in men but not in women. The incidence of colorectal cancer increased 5.51% in men but not in women.

The incidence of stomach and esophagus cancers in men and women decreased in Changle of China from 1988 to 2002, suggesting that the decreased incidence of these tumors is due to the development of economy and effect of tumor prevention in Changle. Epidemiological studies have shown that economic level and dietary habits are

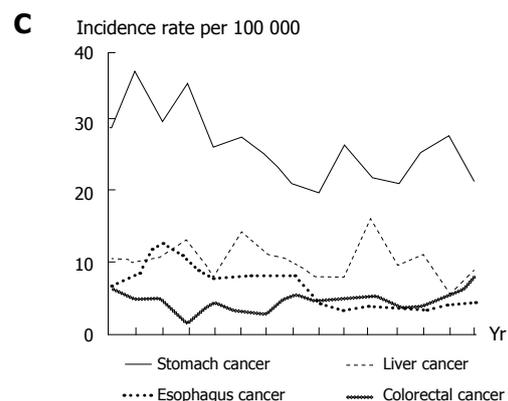
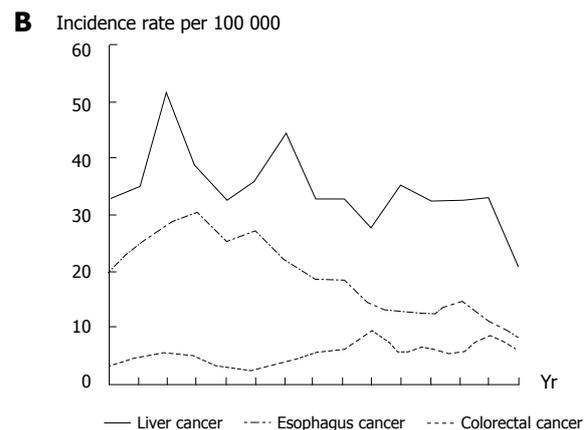
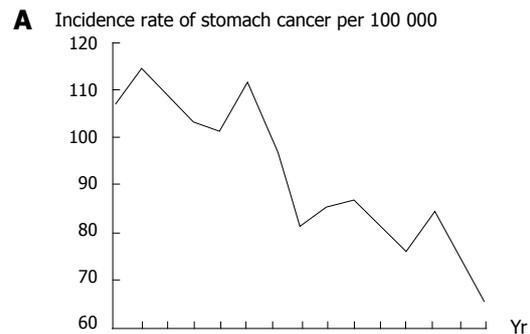


Figure 1 Incidence of stomach cancer (A) and liver, esophagus, colorectal cancer (B) in men, and four tumors (C) in women of Changle from 1988 to 2002.

associated with the incidence of stomach and esophagus cancer^[2,5-7]. Stomach cancer is the most common cancer in Japan and the Japanese have the highest mortality and incidence of stomach cancer in the world. The incidence and mortality of stomach cancer have declined in Japan^[4,8]. The incidence of stomach cancer in a region is related to both economic level and dietary habits. Before 1980s, people in Changle of China had a low living standard and were accustomed to eating salted food and low protein and vitamin C diet. As the economical level is raised and knowledge of tumor prevention is spread in Changle, the people have changed their dietary habits which plays an important role in decreasing the incidence of stomach and esophagus cancer. During 1988-2002, stomach cancer incidence decreased about 38.73% in males and 26.84% in females, and esophagus cancer incidence decreased about 58.41% in males and 29.87% in females in Changle of China. However, stomach cancer is still the most

common cancer in Changle and its incidence in males is still the highest in China although the disease incidence is declined. Therefore, research on preventive factors, such as eating habits, foods, additives, drug use, prevalence of *Helicobacter pylori* infection and environment pollution, is needed.

Our study showed that the digestive system cancer incidence was different in Changle. The incidence of stomach and esophagus cancer declined while colorectal cancer incidence increased in Changle, which is in accordance with the incidence in Japan^[11]. Colorectal cancer incidence tends to increase in males of Changle, and the incidence of the disease in males in 2002 was 2.12 times higher than that in 1988. Colorectal cancer is common in many developed countries^[12,13]. Countries where the people have a high fat intake also have high colorectal cancer incidence^[14]. High fat intake is a risk factor for colon polyp which may progress to colorectal cancer^[15], and 15%-25% of colorectal cancer cases may be attributable to high fat intake^[16]. Moreover, high vegetable and fruit consumption and less meat consumption are associated with a reduced incidence of colorectal cancer^[17-19]. Therefore, a variety of dietary interventions may have a positive impact on dietary behaviors associated with cancer risk^[20].

During 1988-2002, the incidence of liver cancer in males and females were 34.66% and 10.19% in Changle, respectively. It was reported that the incidence of liver cancer is decreased^[21] and liver cancer incidence is increased in China^[22,23].

The incidence of digestive system cancer is different in different areas of China. The reasons for the declined incidence remain unclear. Elimination of HBV-cofactors is likely to contribute to it. However, some risk factors for liver cancer, including smoking and alcohol consumption, are still highly prevalent in males in Changle of China. Therefore, our results might be affected by the lowest incidence in 2002. To confirm whether liver cancer incidence is decreased, further observations are required.

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RAPID COMMUNICATION

Preventive effects of chitosan on peritoneal adhesion in rats

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Abstract

AIM: To study the effects of chitosan gel and blending chiton/gelatin film on preventing peritoneal adhesion in rats.

METHODS: SD rats were randomly divided into 2 groups, group A treated with chitosan gel and group B with blending chiston/gelatin film. In group A, rats were randomly subdivided into 3 subgroups as groups A1, A2 and A3, and different methods were used to induce peritoneal adhesions at the dead end of vermiform process in each group as follows: Group A1 with trauma, A2 with talc powder and A3 with ligation of blood vessel. In each subgroup, rats were redivided into control group and experimental group whose treated vermiform processes were respectively coated with chitosan gel and normal saline immediately after the adhesion-induced treatments. In group B, all the rats received traumatic adhesion-induced treatments and then were randomly divided into 4 groups (groups B1, B2, B3, B4). Group B1 served as control group and were coated with normal saline in the vermiform processes immediately after the treatments, and groups B2, B3 and B4 with 100% chitosan film, chitosan film containing 10% gelatin and chiston film containing 50% gelatin, respectively. At 2 and 4 wk after the above treatments, half of the rats in each terminal group were belly opened, and the peritoneal adhesive situation was graded and histopathological changes were examined.

RESULTS: (1) In group A, regarding peritoneal adhesion situation: At both 2 and 4 wk after the treatments, for groups A1 and A3, the adhesive grades of experimental groups were significantly lower than those of the control group (2 wk: $H = 4.305$, $P < 0.05$ for A1, $H = 6.743$, $P < 0.01$ for A3; 4 wk: $H = 4.459$, $P < 0.05$ for A1, $H =$

4.493, $P < 0.05$ for A3). However, of group A2, there was no significant difference between the experimental and control groups (2 wk: $H = 0.147$, $P > 0.05$; 4 wk: $H = 1.240$, $P > 0.05$). Regarding pathological changes: In groups A1 and A3, the main pathological change was fibroplasia. In group A2, the main changes were massive foreign-body giant cell reaction and granuloma formation with fibroplasia of different degrees. (2) In group B, regarding degradation of film: With increase of the blended gelatin concentration, degrading speed of the film accelerated significantly. Regarding peritoneal adhesion situation: At both 2 and 4 wk after the treatments, the adhesive grades of B1 were the lowest among the four subgroups of B (2 wk: $H = 29.679$, $P < 0.05$; 4 wk: $H = 18.791$, $P < 0.05$). At 2 wk after the treatments, the grades of group B2 were significantly lower than that of groups B3 and B4 ($H = 4.025$, $P < 0.05$ for B2 vs B3; $H = 4.361$, $P < 0.05$ for B2 vs B4). At 4 wk, there were no significant differences of the grades between groups B2, B3 and B4. Regarding pathological changes: Inflammatory cell infiltration and fibroplastic proliferation were observed in the local treated serous membranes, which was the mildest in group B1. Slight foreign-body giant cell reactions were also found in groups B2, B3, and B4.

CONCLUSION: (1) Chitosan gel has preventive effect on traumatic or ischemic peritoneal adhesion, but no obvious effect on foreign body-induced peritoneal adhesion. (2) Chitosan film may exacerbate the peritoneal adhesion. Blending with gelatin to chitosan film can accelerate the degradation of the film, but can simultaneously facilitate the formation of peritoneal adhesion.

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Key words: Chitosan; Gelatin; Peritoneal adhesion; Rat

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INTRODUCTION

Chitosan is the derivant of the chitin after deacetylation, and chitin is a main ingredient of the arthropod shells (such as shrimps, crabs and insects, etc), which is a kind of renewable natural resources and profuse in amount.

Since chitosan is innocuous, biodegradable and with ideal biocompatibility, it has been applied to develop biomaterials^[1,2]. In medical field, chitosan has antiseptic function and can facilitate the healing of wound. It has been widely studied on its potential use to be medical biomaterials. Because of its inhibitory effect on fibroblast growth and the function of mechanical isolation^[3], chitosan has been regarded highly in the prevention of peritoneal adhesion. To evaluate the effects of different types of chitosan on preventing traumatic peritoneal adhesion, a control study in rats was done in this paper.

MATERIALS AND METHODS

Experimental animals and grouping

Two hundred and forty SD rats, 120 females and 120 males, weighing 200-250 g, were provided by the Laboratory Animal Center of Zhejiang Province. They were randomly divided into group A (144 rats) and group B (96 rats). Group A was treated with chitosan gel and group B with blending chiton/gelatin film. In Group A, 144 rats were randomly divided into 3 subgroups (groups A1, A2, A3) with 48 rats in each. Different methods were used to induce peritoneal adhesion in the dead ends of the vermiform processes as follows: group A1 with trauma, group A2 with talc powder and group A3 with ligation of blood vessel. For each subgroup, 48 rats were randomly redivided into control group and experimental group with 24 rats in each. In group B, all the 96 rats received traumatic adhesion-induced treatments as group A1 and then were randomly divided into 4 groups (groups B1, B2, B3, B4) with 24 rats for each. Group B1 served as control group and groups B2, B3, and B4 served as experimental groups treated with 100% chitosan film, chitosan film containing 10% gelatin and chiston film containing 50% gelatin, respectively.

All the rats were fed under the same condition: 24°C -26°C of environmental temperature, about 40% of humidity, alternating 12 h light/dark cycle, free access to food and water.

Surgical methods: Under general anaesthesia with intraperitoneal injection of 3% amobarbital (60 mg/kg), the rats were immobilised in dorsal position, routinely degermed, abdominally incised through a median incision of 2-3 cm long, and the vermiform processes were searched and pulled out of the incision, then the terminal vermiform processes within a distance of 3 cm were treated as follows: In group A1, the anterior surface of serous membrane was scraped slightly with surgical blade till obvious congestion and small bleeding drops appeared. In group A2, talc powders were evenly smeared over the anterior surface of serous membrane. In group A3, the vermiform artery stem was ligated with No 0 surgical thread at the point of 3 cm from the dead end in the following way: loosely knotting the first loop, thrilling a thread with equivalent diameter to the vermiform artery stem through the first loop, tightening the first loop, knotting and tightening the second loop of the ligation knot, and pulling out the thrilled thread. The ligation resulted in a stricture of vermiform artery which induced ischemia of the distal vermiform tissue from the

ligation point. This method had been proved successful in inducing peritoneal adhesion in our former experiments. After the above treatments, for the experimental groups, the treated serous membranes within 3 cm distance from the dead ends were coated with chitosan gel in a dosage of 0.5 mL for each, and the vermiform processes were put back into the abdominal cavities, which were then closed. For the control groups, all the treatments were the same as those of the experimental groups except that the chitosan gel was replaced by normal saline. The duration from opening to closing the abdominal cavity was 5 min, so that the duration of exposure of intestines to air was the same for each rat. In group B, of all the rats, the anterior surfaces of vermiform processes were scraped slightly with surgical blade just as group A1, and the scraped surfaces of B2, B3, B4 were coated with films containing different percentages of gelatin at 0%, 10%, and 50% respectively. Group B1 was treated with normal saline as control. The duration from opening to closing the abdominal cavity was controlled just for 5 min.

At 2 and 4 wk after the surgery, 12 rats (6 females and 6 males) in each subgroup were randomly selected respectively for further study. The abdominal cavity was reopened under anaesthesia, and the grades of peritoneal adhesion were evaluated which existed between the treated vermiform processes and intestines, mesenteria and abdominal walls. After that, the vermiform processes with adhesions were resected, fixed with formalin and histopathologically examined.

Grading standard for the peritoneal adhesion

Referring to Phillips'^[4] grading method of 5 levels and considering the characteristics of peritoneal adhesion in rats, we offered the following grading standard: Grade 0: no adhesions; Grade I: the ratio of adhesive area/the total treated area in the vermiform processes < 20%; Grade II: the ratio is about 40% Grade III: the ratio is about 60%; Grade IV: the ratio is \geq 60%. Each rat was graded by three referees blindly and the average grade of the three was accepted as the adhesive grade of the rat.

Statistical analysis

The H-test of non-parametric statistics for ranked grouped data was used to analyze the differences of the peritoneal adhesive grades between the experimental and control groups. $P < 0.05$ was taken as significant.

RESULTS

Group A

Observation with naked eyes: The skin incisions of all rats healed in first grade. No obvious infection appeared in the abdominal cavity in all rats. In the experimental groups, no obvious residual of chitosan gel could be found at 2 wk after surgery.

Comparison of peritoneal adhesion level: As it shows in Table 1, in groups A1 (trauma-induced adhesion) and A3 (ischemia-induced adhesion), peritoneal adhesion grades of the experimental groups were significantly lower than those of the control groups ($P < 0.05$ or $P < 0.01$) both at 2 and 4 wk after the surgical treatments. While in

Table 1 Comparison of peritoneal adhesion between experimental and control groups in groups A1, A2, and A3

Group		Experimental group (total <i>n</i> = 12)					Control group (total <i>n</i> = 12)					<i>H</i>	<i>P</i>
		0	I	II	III	IV	0	I	II	III	IV		
A1	2 wk	1	7	4	0	0	5	6	1	0	0	4.305	< 0.05
	4 wk	2	7	3	0	0	6	6	0	0	0	4.459	< 0.05
A2	2 wk	0	1	1	4	6	0	0	2	3	7	0.147	> 0.05
	4 wk	0	0	1	2	9	0	0	0	1	11	1.240	> 0.05
A3	2 wk	0	5	5	2	0	2	8	2	0	0	6.743	< 0.01
	4 wk	2	5	4	1	0	6	5	1	0	0	4.493	< 0.05

Table 2 Comparison of peritoneal adhesion in group B rats

Group	<i>n</i>	2 wk (<i>n</i>)					4 wk (<i>n</i>)				
		0	I	II	III	IV	0	I	II	III	IV
B1	24	1	7	4	0	0	2	7	3	0	0
B2	24	1	3	3	4	1	0	3	3	5	1
B3	24	0	0	2	4	6	0	2	2	3	5
B4	24	0	0	0	2	10	0	2	1	5	4
<i>H</i>		29.679					18.791				
<i>P</i>		< 0.05					< 0.05				

group A2 (talc powder-induced adhesion), there was no significant difference in the peritoneal adhesion between the experimental and control groups ($P > 0.05$) both at 2 and 4 wk. The above results indicated that chitosan gel has perfect effect on preventing peritoneal adhesion induced by trauma and ischemia, but no obvious effect on adhesion induced by talc powder.

Comparison of pathological changes: In group A1, at 2 wk after the surgical treatments, there existed obvious fibroplasia and diffused lymphocytes infiltration in the treated serous membrane of the vermiform processes. While at 4 wk after the treatments, the main pathological change was fibroplasia. The above pathological changes in the experimental group were obviously milder than those in the control group. In group A2, at 2 wk, massive foreign-body giant cell reaction (FBGCR) and granuloma formation appeared in the treated vermiform processes with diffuse inflammatory cell infiltration. At 4 wk after the surgical treatment, foreign body granuloma and fibroplasia reaction aggravated with crystal appearing in the foreign-body giant cell. There was no significant difference in the adhesive severity between the experimental and the control groups. In group A3, 2 wk after the surgical treatments, full-thickness fibroplasias and lymphocyte infiltration appeared in the vermiform processes which was more severe in the serous layer. While at 4 wk after the treatment, the lymphocyte infiltration became lighter, but the fibroplasias aggravated obviously. The above pathological changes in the experimental group were obviously milder than that in the control group 2 wk after the treatment. However, at 4 wk, there was no significant difference between the experimental and control groups.

Group B

Observation with naked eyes: The skin incisions of all

rats healed in first grade. No obvious infection appeared in the abdominal cavities at 2 wk after the treatments in all rats. Chitosan film degradation: In group B2, no obvious changes occurred in the chitosan film at 2 wk, and even no obvious rupture could be found in the film at 4 wk after the surgical treatments. The films were only swollen and thinned at 4 wk. In group B3, the films with 10% gelatin were swollen at 2 wk, and degraded into fragments at 4 wk after the treatments. In group B4, the films with 50% gelatin were broken into pieces at 2 wk, and disappeared at 4 wk after the surgical treatments.

Comparison of peritoneal adhesion degree: As listed in Table 2, both at 2 wk and 4 wk after the surgical treatments, there existed significant differences among the four groups ($P < 0.05$) in the peritoneal adhesive degree. At 2 wk, all the differences were significant between every 2 groups ($P < 0.05$) except between groups B3 and B4. At 4 wk, there were significant differences between groups B1 and B2, B1 and B3, B1 and B4 ($P < 0.05$), but there was no significant difference between groups B2 and B3, B2 and B4, B3 and B4 ($P > 0.05$). All the above results indicated that both pure chitosan film and blending chiston/gelatin films could exacerbate peritoneal adhesion, as well as the blended gelatin.

Comparison of pathological changes: (1) At 2 wk after the surgical treatments, the adhesive vermiform processes of all rats were mainly with various degrees of edema, dilation and congestion of capillaries, infiltration of inflammatory cell and fibroplasias. The reaction became severer in the serous membrane than in other layers. The reactions in groups B2, B3, and B4 were more severe than those in group B1. There were slight foreign-body giant cell reaction in groups B2, B3 and B4 (Figure 1). (2) At 4 wk after the treatments, the acute inflammatory reaction decreased obviously in all groups except group B1. In all the other groups (B2, B3 and B4), the fibroplasia was aggravated obviously which caused formation of complete fibrous capsules around the implantation, with a thickest capsule wall in group B4 and a thinnest one in group B2. Furthermore, groups B2, B3, and B4 had obvious foreign-body giant cell reaction (Figure 2).

DISCUSSION

Peritoneal adhesion is an inevitable phenomenon of the natural repairing processes after peritoneal injury. In recent years, many researches have been done on the process and mechanism of peritoneal adhesion. To understand the

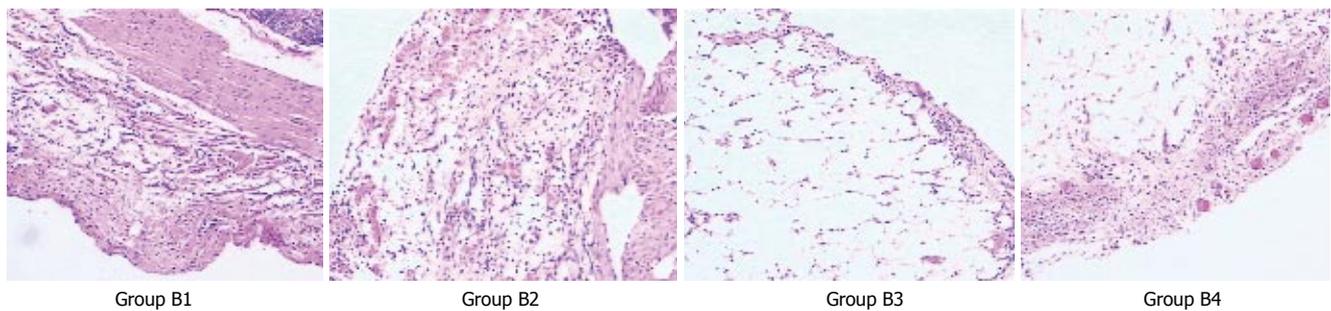


Figure 1 Histopathologic changes of the four B-groups at 2 wk after the surgical treatment.

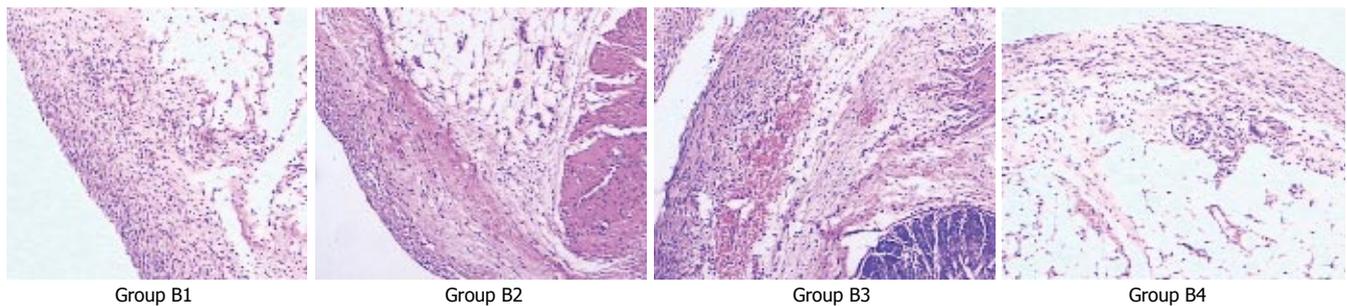


Figure 2 Histopathologic changes of the four B-groups at 4 wk after the surgical treatment.

mechanism of peritoneal adhesion is very important for the prevention of its formation.

Animal studies indicated that the serous membrane injury caused by mechanical injury, ischemia of tissue, stimulation of foreign body and peritonitis was the main reason of peritoneal adhesion. However, whether it results in permanent fibrinous adhesions or not depends on the integrity of the fibrinolytic system^[5,6]. Generally speaking, the formation of peritoneal adhesion needs the following steps: step 1 is the formation of fibrinous gelatinous matrix. This happens within 3 h after the injury. The gelatinous matrix locates among the injured peritoneal membranes, which is the initiation of peritoneal adhesion. Step 2 occurs between 1-5 d after the injury. The fibrinous matrix is gradually replaced by the vascular granular tissues which contain fibroblasts, macrophagocytes and giant cells. Most of the fibrins disappear and are replaced by a large number of fibroblasts and collagen fibers, and then the fibrin network is formed. Step 3 takes place during 5-10 d after the injury. The fibroblasts acquire a regular alignment gradually and the collagen deposition is enriched. Step 4 is at 2 wk after the injury. The component cells, especially the fibroblasts, decrease obviously and are covered by mesothelial cells. Eventually, the fibrinous adhesions are formed. However, after peritoneal injury, the fibrin can be decomposed by fibrinolysin into fibrin degradation products (FDPs) in the course of fibrinous gelatinous matrix formation, which is unrelated with peritoneal adhesion. It generally occurs at 72-76 h after the injury. In brief, when peritoneal injury occurs, whether the wounds heal through adhesive fusion or through epithelization, mainly depends on the degree of local fibrinolysis and whether epilethelial juxtapposition exists^[7].

Based on how and why the peritoneal adhesion happens, more than 10 preventive methods have been proposed. Among them, mechanical isolation seems to have the brightest prospect. At present, close attention has been paid to the following 4 materials which are used as mechanical isolation: EPTEE, oxidized regenerated cellulose (ORC), HA-CMC and chitosan^[8,9]. Chitosan is highly regarded as a biomaterial for prevention of peritoneal adhesion, because it has the functions of anti-infection, hemostasis, inhibiting growth of fibroblasts, mechanical isolation and moreover, it is biodegradable.

Chitosan is the derivant of deacetylated chitin. Chitin was discovered in 1811. Its molecular structure was determined by chemical method and X-ray diffraction in 1887. Chitin can be converted into chitosan after deacetylation. Chitosan is soluble, easily to be chemically modified because it contains many amid and hydroxyl. Meanwhile, this kind of natural polysaccharose is alkaline and has good biocompatibility and is biologically degradable. Its degradation products are acetylglucosamine and aminoglucose, which are atoxic for human bodies. The low molecular weight of chitosan and its oligosaccharide produced during degradation render it to have no immunogenicity and not to accumulate *in vivo*. The biological activity of chitosan mainly includes: (1) inhibiting the growth of bacteria and mold; (2) antineoplastic activity: It can selectively agglutinate the L1210 cells in leukemia and Ehrlich's cells in carcinomatous ascites, but does not affect the normal erythroid bone marrow cells; (3) immunological enhancement: It can efficaciously enhance the function of macrophages and the activity of hydrolase, stimulate the macrophage to produce lymphokine and initiate the immune response. But it cannot promote the production

of antibody; (4) anticoagulant activity: The chitosan after thioesterification has a similar chemical structure with heparin so that it has a good anticoagulant activity; (5) promoting tissue repair and hemostasis: Since the degrading product of chitosan is charged, it can induce platelet aggregation and activate the coagulation system. Chitosan can inhibit fibrous hyperplasia during wound healing and efficaciously enhance wound healing. Animal experiments in recent years have proven that chitosan gel has good effect in preventing peritoneal adhesion^[3,4].

All the 3 different animal models of peritoneal adhesion used in our study were monofactor-induced peritoneal adhesion, which made the study of effects of chitosan on preventing peritoneal adhesion due to different causes technologically possible. Observation at 2 and 4 wk after the surgical treatments in all the control groups showed that the incidence of peritoneal adhesion was 90.9% and 83.3% respectively, and most of the adhesions were graded as I and II. This proved that the animal model was reliable and stable.

In our study, different chitosan materials were used for the prevention of peritoneal adhesion, which turned out to have completely different results. Because the peritoneal adhesions in each group were caused by different methods, different preventing effect was observed. In group A (treated with chitosan gel), the chitosan gel film had satisfactory effects on the prevention of peritoneal adhesion due to injury or ischemia. At 2 wk after the treatments, the chitosan gel was completely biologically degraded. Moreover, the peritoneal adhesion level and pathological change in both groups A1 and A3 were milder than those in the control groups. This indicated that the chitosan gel had an evident preventing effect against peritoneal adhesion due to injury or ischemia. In group A2 (adhesions induced by talc powder), both histopathologic examination and adhesion grades indicated that the chitosan gel had no evident preventing effects on peritoneal adhesion induced by talc powder. The probable cause may be related with the pathological change in adhesions induced by talc powder, which mainly was foreign body granuloma reaction with massive fibroplasias. As long as the foreign body existed, the foreign body granuloma and fibroplasia reaction would remain persistent. Since the chitosan gel could be fastly degraded *in vivo* (within 2 wk after surgery), the acting time of the chitosan gel was too short to prevent against persistent foreign body granuloma reaction.

Chitosan gel is still imperfect because it is highly flowable. When chitosan gel is smeared at the surface of wounded peritoneum, it cannot come to a high concentration in the focus, resulting in weakened effect since its acting time is shortened. In order to improve the concentration of chitosan in the intraabdominal focus and make a more thoroughly mechanical isolation, some researchers used pure chitosan film instead to get a better effect on peritoneal adhesion prevention. However, our study indicated that such effort was disappointing.

In Group B2 (treated with pure chitosan film), at 4 wk after the treatments, the chitosan film was still undegraded. Such slow degrading speed was detrimental for an anti-peritoneal adhesion material because if the postsurgical

initial membranous adhesions cannot be degraded in time, it will form irreversible fibrinous adhesions, which cannot be inhibited by chitosan. On the contrary, the intraabdominal residual of undegraded chitosan film can evoke the foreign body reaction and result in fibrous capsule formation, which facilitates the formation of peritoneal adhesion. These were proved in our study: the adhesion grade in group B2 was higher than that in control group, and histopathologic examination indicated obvious foreign-body giant cell reaction at 2 and 4 wk after the surgical treatments.

Because the slow degradation of chitosan film weakened its anti-adhesive function, blending chiston/gelatin film was suggested to improve the *in vivo* degradation speed of chiston film. And experiments proved that the more gelatin added in the film, the more fastly the blending film *in vivo* degraded. However, there have been no experimental data about the effect of the blending chiston/gelatin film on the prevention of peritoneal adhesion.

Gelatin is a polypeptide mixture which is soluble in hot water. It is widely used in medical field as haemostat and dermagraft and dressing materials of wounds because it can be degraded quickly *in vivo*. In our study, the blending chiston/gelatin films with different concentration of gelatin were used in group B2 (10% gelatin) and B4 (50% gelatin), and the results indicated that the higher concentration of gelatin in the film, the more fastly it was degraded in the abdominal cavity of rats. However, our study indicated that the anti-peritoneal adhesion effect of the blending film was not ideal. The peritoneal adhesion grade was not only lower than that of the control group, but also lower than that of group B2 (with pure chiston film). The probable reasons are: although the blending chiston/gelatin film had a faster degradation speed, the film still could not be completely degraded even at 4 wk after the treatments in group B3 and at 2 wk in group B2. The residual blending film may cause foreign body reaction. In addition, gelatin is a polypeptide mixture which is probably antigenic and can cause immunological rejection. This in turn promotes and facilitates the formation of local peritoneal adhesions.

We conclude that: (1) Chitosan gel has perfect effect on preventing peritoneal adhesion due to injury or ischemia, but no evident effect on peritoneal adhesion induced by talc powder; (2) Pure chitosan film could not prevent peritoneal adhesion because of low *in vivo* degradation speed; (3) Chitosan film blended with gelatin could exacerbate peritoneal adhesion.

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RAPID COMMUNICATION

Anti-inflammatory effect of Diammonium Glycyrrhizinate in a rat model of ulcerative colitis

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Abstract

AIM: To explore the anti-inflammatory mechanism of Diammonium Glycyrrhizinate in a rat model of ulcerative colitis induced by acetic acid.

METHODS: Sprague-Dawley female rats were divided into four groups: Diammonium Glycyrrhizinate group, dexamethasone group, acetic acid control and normal control group. Colonic inflammation was evaluated by disease activity index, gross morphologic damage, histological injury and colonic myeloperoxidase activity. Immunohistochemistry was used to detect the expression of NF- κ B, TNF- α and ICAM-1 in colonic mucosa.

RESULTS: Compared to the acetic acid control, both Diammonium Glycyrrhizinate and dexamethasone showed a significant anti-inflammatory effect ($P < 0.01$). The expression of NF- κ B, TNF- α and ICAM-1 in colonic mucosa was significantly lower in the Diammonium Glycyrrhizinate group and dexamethasone group than in the acetic acid group.

CONCLUSION: Diammonium Glycyrrhizinate could reduce inflammatory injury in a rat model of ulcerative colitis. This may occur via suppression of NF- κ B, TNF- α and ICAM-1 in colonic mucosa.

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Key words: Ulcerative colitis; Diammonium Glycyrrhizinate; Mechanism

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inflammatory effect of Diammonium Glycyrrhizinate in a rat model of ulcerative colitis. *World J Gastroenterol* 2006; 12(28): 4578-4581

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INTRODUCTION

Traditional drugs for ulcerative colitis, though quite effective, may exert severe side-effects. New therapeutic agents with less side-effects would be very useful. Diammonium Glycyrrhizinate (DG) is a substance that is extracted and purified from a traditional Chinese medicinal herb. The herb has been used traditionally for the treatment of hepatitis due to its anti-inflammatory effect, resistance to biologic oxidation, membranous protection and a weak steroidal action. However, it was not known whether DG might also be effective in other inflammatory conditions, such as ulcerative colitis. To explore a possible anti-inflammatory effect of DG in ulcerative colitis, the expression of NF- κ B, TNF- α and ICAM-1 in colonic mucosa was detected by immunohistochemistry in a rat model.

MATERIALS AND METHODS

Materials

Female SD rats were purchased from the Center of Experimental Animals in the 89th Hospital of PLA. NF- κ B, TNF- α and ICAM-1 antibodies and immunohistochemistry kits were purchased from Beijing Zhongshan Biotech Company. MPO kit was purchased from the Institute of Nanjing Jiancheng Biotechnology. DG was purchased from Jiangsu Chia Tai Tianqing Pharmaceutical Co., LTD (X20010756, DG0267).

Methods

Preparation of animal model: Forty SD rats were divided into four groups: DG group, dexamethasone group, acetic acid control and normal control group. All rats were fasted for twenty-four hour. Before the colonic infusion of acetic acid, 0.3 mL (30 mg/kg) Natrium pentobarbital was injected peritoneally. A polyethylene catheter was put into the colon extending a distance of eight centimeters beyond the anus. For DG, dexamethasone, and the acetic acid control groups, 1 mL of 10% (v/v) acetic acid was

Table 1 Morphologic injury scoring system

Morphologic injuries	Score
Local edema and congestion without ulcer	1
One ulcer without congestion or thick colonic wall	2
One ulcer with inflammation	3
Two ulcers, lesion diameter less than 1 cm	4
More than two ulcers with or without inflammation, lesion diameter 1-2 cm	5
Lesion diameter over 2 cm, one more score for one additional 1 cm increasing diameter	6-8

infused into the colon through this catheter^[1], held in place for 30 s, and then flushed with 5 mL normal saline. Only normal saline was infused into the colon in the normal control group. In the DG group, 40 mg/kg DG was injected intraabdominally every day for one week; in dexamethasone group, 0.2 mg/kg dexamethasone was injected intraabdominally cavity daily for one week; in the acetic acid control and normal control groups, equal volumes of normal saline were injected into the abdominal cavity daily for one week.

Preparation of colonic tissue samples: Seven days after acetic acid infusion, the body weight of each rat was measured and recorded. The distal colon was removed at the level of 8 cm from the anus. Samples were evaluated by morphological scores, and then cut into two parts-one for H&E staining and immunohistochemistry, the other for the detection of MPO activity.

Evaluation of colonic inflammation: Colonic inflammation was evaluated seven days after acetic acid infusion by disease activity index (DAI), morphologic injury, histological changes, and MPO activity. DAI was calculated as the sum of scores assigned as follows: percentage of body weight reduction (0: no change, 1: 1%-5%, 2: 6%-10%, 3: 11%-15%, 4: > 15%), Stool consistency (0: normal, 2: loose, 4: diarrhea) and the presence of fecal blood (0: normal, 2: positive occult blood test, 4: visible bleeding)^[2]. Morphologic injury was evaluated using the scoring system reported by Dieleman, with slight modifications^[3] (Table 1). Ten fields were selected randomly to estimate histological injury according to the scoring system listed in Table 2^[4]. A kit was used to detect MPO activity in the colonic mucosa.

Immunohistochemical detection of NF- κ B p65, TNF- α and ICAM-1: The expression of NF- κ B p65, TNF- α and ICAM-1 in colonic mucosa was detected by a SP immunohistochemistry kit. On each slide, five fields (\times 400) were selected randomly and the percentage of positive cells was counted among 1000 cells. The average of positive cells in five fields was the determined.

Statistical analysis

Data are presented as mean \pm SD, and statistical differences among groups were tested using the SPSS statistical software package. Based on the result of a test of homogeneity of variances, one-way ANOVA and SNK-q test were applied to test differences between different groups. A *P* value of 0.05 was set as the level of statistical significance.

Table 2 Histological scoring system

Histological changes	Score
Normal colonic mucosa	0
Cryptal defect less than 1/3	1
Cryptal defect 1/3-2/3	2
Slight inflammatory infiltration in proper lamina	3
Mucosal erosion or ulcer with significant inflammatory infiltration	4

Table 3 DAI, morphologic injury, histological changes, and MPO activity in rats assigned to the different treatment groups

Group	DAI	Morphologic injury	Histological changes	MPO activity (U/g tissue)
DG	3.10 \pm 0.54 ^{b,d,f}	2.80 \pm 0.76 ^{b,d,f}	3.22 \pm 0.88 ^{b,d,f}	0.67 \pm 0.06 ^{b,d,f}
Dexamethasone	1.90 \pm 0.43 ^{b,d}	1.70 \pm 0.54 ^{b,d}	1.76 \pm 0.59 ^{b,d}	0.38 \pm 0.05 ^{b,d}
Acetic acid control	6.70 \pm 0.84 ^b	6.30 \pm 0.95 ^b	5.96 \pm 0.97 ^b	1.32 \pm 0.09 ^b
Normal control	0.60 \pm 0.38	0.60 \pm 0.52	0.74 \pm 0.56	0.19 \pm 0.05

^b*P* < 0.01, vs Normal control group; ^d*P* < 0.01, vs Acetic acid control group; ^f*P* < 0.01, vs Dexamethasone group.

RESULTS

DAI, morphologic injury, histological changes, and MPO activity

1-2 d after colonic infusion of acetic acid, rats displayed diarrhea, pyemic stool, and reduced body weight. Morphologically, a dilated lumen, thickened wall, and brown or black color was observed continuously in the injured bowel. Edema, erosions, necrosis, superficial ulcerations, crypt abscesses, and inflammatory infiltration into the lamina propria were observed in the injured segment by light microscopy. In Table 3, according to DAI, scores of morphological and histological changes, and MPO activity, the colon showed significant pathogenic changes in the DG, dexamethasone, and acetic acid control groups compared to the normal control group that received saline alone (*P* < 0.01), which demonstrated that acetic acid infusion results in injuries that are comparable to those seen in humans with ulcerative colitis. These inflammatory indices were significantly improved by DG and dexamethasone (*P* < 0.01). The anti-inflammatory effect of DG was significantly lower than that of dexamethasone (*P* < 0.01).

Expression of NF- κ B p65, TNF- α and ICAM-1 in injured colon

In rats that received acetic acid, NF- κ B p65 was positive mainly in nuclei of most endothelial cells, epithelial cells and mononuclear cells, especially in the mucosa and submucosa. TNF- α and ICAM-1 were positive mainly in the cytoplasm, membrane and rarely in nuclei. ICAM-1 was positive in most endothelial cells and macrophages. TNF- α positive cells, including mononuclear cells, macrophages and neutrophils, were located densely in lamina propria and in proximity to the muscularis. The percentage of cells positive for these three molecules was significantly correlated

Table 4 Expression of TNF- α , ICAM-1 and NF- κ B p65 in injured colon

Group	TNF- α		ICAM-1		NF- κ B p65	
	Percentage	Density	Percentage	Density	Percentage	Density
DG	32.2 \pm 8.2 ^{b,d,f}	37.3 \pm 7.0 ^{b,d,f}	34.3 \pm 8.2 ^{b,d,f}	36.1 \pm 6.1 ^{b,d,f}	23.3 \pm 5.2 ^{b,d,f}	31.2 \pm 7.8 ^{b,d,f}
Dexamethasone	17.9 \pm 5.6 ^{b,d}	19.0 \pm 5.2 ^{b,d}	18.7 \pm 5.7 ^{b,d}	18.5 \pm 5.2 ^{b,d}	15.5 \pm 4.3 ^{b,d}	17.6 \pm 4.9 ^{b,d}
Acetic acid control	52.5 \pm 9.1 ^b	74.1 \pm 9.5 ^b	60.2 \pm 8.3 ^b	70.7 \pm 9.7 ^b	44.5 \pm 8.9 ^b	51.5 \pm 9.8 ^b
Normal control	7.6 \pm 5.7	9.0 \pm 4.8	9.1 \pm 4.4	9.4 \pm 4.9	7.6 \pm 4.1	8.1 \pm 4.2

^b $P < 0.01$, vs Normal control group; ^d $P < 0.01$, vs Acetic acid control group; ^f $P < 0.01$, vs Dexamethasone group.

ted with the degree of inflammatory injury (Table 4), and these markers were rarely expressed in samples taken from the normal control group. The positive percentage and density of NF- κ B p65, TNF- α and ICAM-1 in injured colon was significantly higher than that in normal control. After DG or dexamethasone treatment, the positive percentage and density of these molecules were reduced significantly, which indicates that both DG and dexamethasone may inhibit the expression of these molecules. Also, the expression of these molecules was significantly lower in DG treated samples than in dexamethasone treated samples ($P < 0.01$).

DISCUSSION

DAI, morphologic injury and histological changes are usually applied to evaluate the severity of inflammatory injuries. MPO reflects the activity of neutrophils, which is also a good indicator of the acute inflammatory reaction^[5,6]. According to the indices mentioned above, a rat model of ulcerative colitis (acute phase) was induced by colonic infusion of acetic acid in these experiments. Compared with the acetic acid control group, these inflammatory indices were significantly improved by treatment with DG or dexamethasone.

To understand the molecular mechanism of the effect of DG in this experimental model, the expression of NF- κ B, ICAM-1 and TNF- α was detected by immunohistochemistry. All of these molecules are known to be upregulated in the acute inflammatory cascade, which plays an important role in the pathogenesis of ulcerative colitis^[7]. Activation of NF- κ B is key to the expression of many proinflammatory cytokines and adhesive molecules including ICAM-1 and TNF- α ^[8-11]. ICAM-1 induces the migration and infiltration of inflammatory cells into the lesion^[12,13], while TNF- α causes apoptosis of the colonic mucosa^[14,15]. Both ICAM-1 and TNF- α represent different key points in the progress of inflammatory injuries^[16,17]. TNF- α , ICAM-1 and NF- κ B were upregulated in the acetic acid control group, but reduced significantly after DG or dexamethasone treatment, which means that inhibition of these molecules was likely important for the protective effect of DG and dexamethasone.

As reported previously, NF- κ B is a key molecule in both the initiation and progression phase of the inflammatory reaction^[18,19]. Activated NF- κ B translocates into the nucleus and induces the expression of proinflammatory cytokines, adhesive molecules and chemokines. In this rat model, the expression of NF- κ B was inhibited by

both DG and dexamethasone, which means that the anti-inflammatory mechanism of DG may be similar to that of dexamethasone. Though the efficacy of DG was less than that of dexamethasone, its side-effects are expected to be less severe.

In summary, DG was efficacious in experimental ulcerative colitis induced in rats, and associated with insignificant side effects. This result suggests that DG may be a promising drug candidate for the treatment of ulcerative colitis.

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RAPID COMMUNICATION

Expression of vascular endothelial growth factor-C and angiogenesis in esophageal squamous cell carcinoma

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Abstract

AIM: To investigate the expression of vascular endothelial growth factor-c (VEGF-C) mRNA and microvessel density (MVD) in human esophageal squamous cell carcinoma (ESCC) and its relationship with clinical significance.

METHODS: Specimens obtained from 43 patients undergoing surgical resection for ESCC were used in this study. The expression of VEGF-C mRNA was examined by *in situ* hybridization. Tumor MVD was determined immunohistochemically with anti-CD31 antibody and estimated by image analysis. Ten sections of adjacent normal mucosa were also examined.

RESULTS: VEGF-C mRNA expression was detected in cytoplasm of carcinoma cells. Of the 43 ESCC patients studied, 18 cases (41.9%) were positive for VEGF-C mRNA. No VEGF-C mRNA expression was observed in normal esophageal mucosa. VEGF-C mRNA expression correlated significantly with lymph node metastasis, TNM stage and depth of invasion ($P < 0.05$). Furthermore, histological grade (differentiation) tended to correlate with VEGF-C mRNA expression, but was not statistically significant ($P > 0.05$). In tumor lesions, the MVD was significantly greater than that in normal esophageal mucosa. MVD correlated significantly with lymph node metastasis, TNM stage and depth of invasion ($P < 0.05$), but not with histological grade (differentiation) ($P > 0.05$). Lesions with VEGF-C mRNA expression had a significantly higher MVD than that of those without VEGF-C mRNA expression ($P < 0.05$).

CONCLUSION: VEGF-C plays a role in lymphatic metastasis via lymphangiogenesis and angiogenesis in ESCC. VEGF-C is one of the important predictors of the biological behavior in ESCC.

INTRODUCTION

Lymph node metastasis is an important prognostic factor for human esophageal squamous cell carcinoma (ESCC)^[1]. Angiogenesis induced by a variety of growth factors such as vascular endothelial growth factor (VEGF), has been recently considered necessary for the growth, invasion and metastasis of solid tumors^[2]. Thus, measurement of vascular growth may be clinically important in esophageal cancer specimens. To assess angiogenesis, several markers of blood vessel endothelium have been developed for microscopic estimation of microvessel density (MVD) by immunohistochemistry, including CD31/PECAM-1, CD34, and factor VIII-related antigen (von Willebrand factor or vWF)^[3]. VEGF-C, a member of the VEGF family, stimulates the proliferation of both vascular and lymphatic endothelial cells via VEGF receptor (VEGFR)-2 and VEGFR-3 in many physiological and pathological processes^[2,4]. Previous reports have shown that VEGF-C expression in cancer tissues has a positive correlation with the risk of lymphatic metastasis in a variety of cancers^[5] including prostatic^[6], gastric^[7], oral^[8], lung^[9], colorectal^[10,11] and bladder carcinoma^[12]. A similar tendency has been reported for esophageal cancers, although a significant correlation between VEGF-C expression and the frequency of nodal metastasis is not always found^[13-15]. Hence, the precise role of VEGF-C in ESCC has not been clearly understood^[1]. Therefore, in the present study, VEGF-C mRNA expression in biopsy specimens of ESCC was examined by *in situ* hybridization (ISH) and the association between VEGF-C mRNA expression and clinicopathological factors, including the intratumoral microvessel density (MVD) as a measure of tumor angiogenesis in ESCC was investigated.

MATERIALS AND METHODS

Patients and tumor specimens

Tumor specimens from 43 ESCC patients (32 males and 11 females, mean age 62.5 years, range 47-76 years) undergoing surgical treatment in Jinhua People's Hospital from 1997 to 2002 were included in the study. Specimens were classified according to the TNM classification system by UICC and 10 normal esophageal tissues were collected as control. None of the patients received any radiotherapy or chemotherapy prior to the study. Histological grades, depth of invasion and lymph node metastasis are shown in Table 1.

Methods

In situ mRNA hybridization (ISH) analysis was performed as described previously with minor modifications^[16]. VEGF-C specific oligonucleotide probes were labeled with digoxin at the 5'-end and synthesized by Beijing Dingguo Co. Ltd (Dingguo, Beijing, China). The DNA oligonucleotide sequences used are 5'-TGTACAAGT GTCAGCTAAGG-3' and 5'-CCACATCTATACACAC CTCC-3'. Tissue sections (4 μ m-thick) were incubated with 50 mg/L proteinase K, and then hybridization was performed in a moist chamber for 16 h at 45°C, using a probe concentration of 100 ng/L. The sections were then incubated with a staining solution containing NBT/BCIP in a dark box for 1-2 h. Negative controls were performed in all cases by omitting the probes.

A mouse CD31 mAb (JC/70A, DAKO Corporation, Denmark) was used as a vascular endothelial marker^[3,17]. Immunohistochemical examination for CD31 was performed with EnVision kits to access MVD. In brief, after deparaffinization and rehydration, the sections were denatured for 3 min in a microwave oven in citrate buffer (0.01 mol/L, pH 6.0) for antigen retrieval. Endogenous peroxidase activity was quenched with hydrogen peroxide, and the sections were incubated with goat serum for 20 min at room temperature to block nonspecific binding of anti-CD31 mAb (JC/70A, 1:50 dilution) and then for 16 h at 4°C at 1:50 dilution. Bound antibodies were detected by the EnVision system (DAKO Corporation, Denmark) and using 3, 3'-diaminobenzidine as the chromogen while nuclei were counterstained with hematoxylin. Negative controls were obtained by omission of the primary antibodies.

The results of ISH and MVD were examined by two of the authors with no prior knowledge of the identification of each section, using Leica Qwin image system (Leica Corporation, Germany). The expression of VEGF-C mRNA in the tissue was defined as positive if distinct staining was observed in at least 10% of tumor cells^[18]. For determination of MVD, the number of microvessels with positive reaction to JC/70A was counted in five randomly selected microcopy fields on the vascular areas within a section ($\times 200$, 0.7386 mm²/field) and the average was then calculated.

Statistical analysis

The data were evaluated by *t* test, Fisher's exact probability

Table 1 Correlation between VEGF-C mRNA expression in ESCC tissue and clinicopathological factors

Clinicopathological factors	<i>n</i>	VEGF-C mRNA <i>n</i> (%)	MVD/mm ² (mean \pm SD)
TNM stage			
I, II	33	11 (33.3)	60.14 \pm 20.2
III, IV	10	7 (70.0) ^a	83.68 \pm 33.6 ^a
Histological grade			
G ₁	12	4 (33.3)	60.5 \pm 31.7
G ₂	20	8 (45.0)	66.30 \pm 21.6
G ₃	11	6 (54.6)	71.66 \pm 30.8
Depth of invasion			
T ₁ , T ₂	23	5 (21.7)	64.24 \pm 19.3
T ₃ , T ₄	20	13 (65.0) ^b	68.86 \pm 31.7
Lymph node metastasis			
Negative	31	10 (32.3)	58.66 \pm 18.8
Positive	12	8 (66.7) ^a	85.30 \pm 32.7 ^a

^a*P* < 0.05 vs negative lymph node metastasis and TNM stages III and IV; ^b*P* < 0.01 vs depth of tumor invasion of T₃ and T₄.

test or chi square test. *P* < 0.05 was considered statistically significant for all tests. All statistical analyses were performed using the SPSS 10.0 statistical package (SPSS, Inc, Chicago, IL).

RESULTS

Expression of VEGF-C mRNA in ESCC tissues

Of the 43 patients studied, 18 cases (41.9%) were positive for VEGF-C mRNA expression in cytoplasm. The specimens were divided into two categories by the staining pattern of VEGF-C mRNA, diffuse or focal staining of carcinoma cells (Figures 1A and 1B). In contrast, no VEGF-C mRNA in specimens of adjacent normal esophageal mucosa was stained.

CD 31 expression and MVD in ESCC tissues

Immunohistochemical expression of CD31 was detected in cytoplasm of vascular endothelial cells. Brown capillaries or small clusters standing out sharply from other tissues were found to be microvessels (Figures 2A and 2B). At the deepest invasive site of tumors, the MVD ranging 43-144/mm² (76.4 \pm 20.3/mm²) was significantly higher than that in normal esophageal mucosa (*P* < 0.01).

Correlation between VEGF-C mRNA expression and MVD and clinicopathological factors

VEGF-C mRNA expression in ESCC tissues was associated with the depth of tumor invasion, TNM stage and lymph node metastasis (*P* < 0.05), but not with histological grade (*P* > 0.05). Furthermore, MVD also correlated with TNM stage and lymph node metastasis in ESCC tissue (*P* < 0.05). No association was found between MVD and depth of tumor invasion and histological grade (*P* > 0.05). The patients were divided into VEGF-C mRNA positive and negative groups. The VEGF-C mRNA positive cases showed a higher MVD (79.5 \pm 30.9/mm²) than the VEGF-C mRNA negative cases (57.5 \pm 18.4/mm²) (*P* < 0.05) (Table 1).

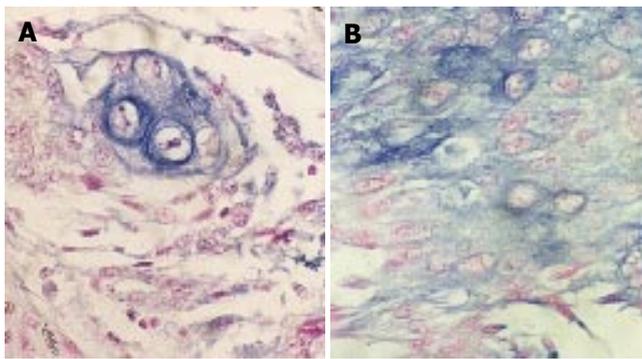


Figure 1 Focal (A) and diffuse (B) expression of VEGF-C mRNA in ESCC tissue as blue granules in cytoplasm of tumor cells (*in situ* hybridization $\times 400$).

DISCUSSION

Recent studies have shown a significant relation between MVD, VEGF expression and prognosis in a variety of tumors including esophageal carcinoma^[19]. Among the VEGF family members, VEGF-C appears to affect the lymphatic system as a ligand for VEGFR-3, which is specifically expressed on lymphatic vessels, and VEGF-C is suspected to play an important role in lymphangiogenesis^[2,4,5]. But little is known about the mechanisms of lymphangiogenesis and lymphatic metastasis, while the effects of VEGF-C in ESCC remain controversial^[1]. In this study, we investigated the clinicopathological significance of VEGF-C mRNA expression in relation to the angiogenesis in ESCC. The results showed that 41.9% of patients with ESCC had expression of VEGF-C mRNA, which correlated significantly with lymph node metastasis, depth of tumor invasion and TNM stage, suggesting that VEGF-C plays a role in ESCC. It was reported that VEGF-C can be detected using immunohistochemical staining and RT-PCR, and VEGF-C expression in ESCC may play a key role in tumor progression and lymphatic metastasis^[13,14]. It was also reported that only histological grade, but not parameters involved in lymphatic spread, correlates with VEGF-C expression in ESCC^[15]. These contradictory findings can be explained by difference in analytic method. Even in immunohistochemical analysis, the result may differ depending on the selected site for assessment.

More interestingly, we found that VEGF-C mRNA expression was significantly correlated with MVD, indicating the grade of angiogenesis in ESCC. Tsurusaki *et al*^[6] and Akagi *et al*^[18] have failed to find any remarkable differences in blood vessel density between VEGF-C positive and negative prostatic and colorectal carcinoma specimens. Also, VEGF-C expression is not correlated with MVD in human gallbladder cancer^[20]. Whereas other studies reported that VEGF-C is associated with angiogenesis in breast cancer^[21], colorectal carcinoma^[22,23] and bladder transitional cell carcinoma^[12], and plays an important role in angiogenesis and lymphangiogenesis in lung cancer^[9] and malignant mesothelioma^[24]. It has been reported that VEGF-C can bind to both VEGFR-2 and VEGFR-3^[25]. The specificity of VEGF-C for its 2 receptor is known to be regulated by proteolytic processing.

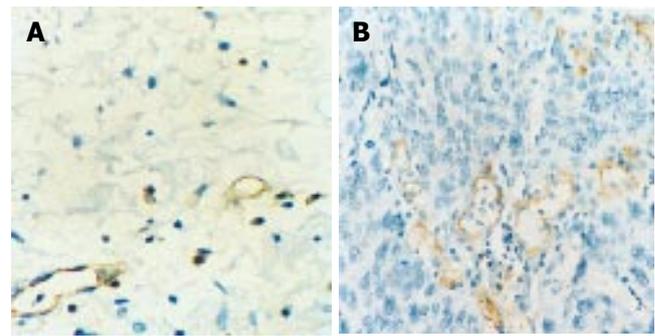


Figure 2 Immunohistochemical staining of CD 31 in normal esophageal (A) and ESCC (B) tissue (EnVision $\times 400$).

Accordingly, only fully processed VEGF-C activates both VEGFR-2 and VEGFR-3, whereas the partially processed precursors act only through VEGFR-3^[26]. Activation of VEGFR-2 results in mitogenesis of vascular endothelial cells, whereas VEGFR-3 activation by VEGF-C is considered to induce proliferation of lymphatic endothelial cells. Furthermore, recent studies have revealed that VEGFR-3 is expressed in angiogenic blood vessels in certain pathological conditions. For example, VEGFR-3 expression can be detected in intratumor blood vessels of human breast cancer^[21], cutaneous melanoma^[27], head and neck squamous cell carcinoma^[28], gliomas as well as in vascular endothelial cells activated during formation of granulation tissue in skin^[29]. Thus, angiogenesis and lymphangiogenesis responses to VEGF-C have been found to depend on the expression of its receptors in blood and lymphatic endothelial cells of the target tissue.

In our present study, patients with VEGF-C mRNA expression had a significantly higher MVD than those without VEGF-C mRNA expression, suggesting that VEGF-C secreted from tumor cells may stimulate not only lymphangiogenesis but also angiogenesis, which are significantly correlated with lymph node metastasis in ESCC. As a rule, once cancer cells enter blood microvessels, they can reach regional lymph nodes through lymphatic vessels from blood vessels, via blood vessel-lymph vessel junctions. In fact, the lymphatic and vascular systems have numerous connections allowing disseminating tumor cells to pass rapidly from one system to the other. Furthermore, metastatic tumor cells expressing VEGF-C could grow in regional lymph nodes that they reached.

In conclusion, VEGF-C mRNA is expressed heterogeneously in tumor cells and plays an important role in angiogenesis and lymphangiogenesis, as well as growth and metastasis of ESCC. The combined examination of VEGF-C expression and MVD in biopsy specimens from ESCC patients can predict lymph node metastasis and select appropriate treatments.

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CASE REPORT

Diagnosis of autoimmune gastritis by high resolution magnification endoscopy

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Abstract

Endoscopic visualisation of gastric atrophy is usually not feasible with conventional endoscopy. Magnifying endoscopy is helpful to analyze the subepithelial microvascular architecture as well as the mucosal surface microstructure without tissue biopsy. Using this technique we were able to describe the normal gastric microvasculature pattern and we also identified characteristic patterns in two cases of autoimmune atrophic gastritis.

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Key words: Magnification endoscopy; Autoimmune gastritis; Collecting venules; Subepithelial capillary network

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INTRODUCTION

Endoscopic visualisation of gastric atrophy is usually not feasible with conventional endoscopy. Except for the absence of rugae and visible vessels in the gastric corpus, macroscopic features, as observed during gastroscopy, are of very limited value in the evaluation of the presence of gastric atrophy^[1]. Although histology may be considered as a gold standard for detection of gastric atrophy, neither the original nor the revised version of the Sydney system reliably identifies more than half of the cases in patients

with confirmed atrophy^[2].

Magnifying endoscopy is a helpful procedure to analyze the subepithelial microvascular architecture as well as the mucosal surface microstructure without tissue biopsy^[3]. Using this technique we were able to observe the normal gastric microvasculature pattern and we also identified characteristic patterns in two cases of autoimmune atrophic gastritis.

CASE REPORT

Two male patients (aged 53 and 62 years), underwent upper gastrointestinal endoscopy due to anaemia and low serum B12 levels. Endoscopy was performed using the GIF-Q240Z (Olympus, Keymed, UK) high-resolution magnifying endoscope ($\times 115$ on a 20" screen, 7.9 μm resolution). Prior to endoscopy, a soft black hood was mounted on the tip of the scope, to enable the endoscopist to fix the focal distance at 2 mm between the tip of the scope and the gastric mucosa. Ordinary endoscopic findings were normal, however, magnified observation of the gastric mucosa showed normal gastric antral microvasculature (coil-shaped subepithelial capillary network (SECN) pattern) (Figure 1A), with disappearance of the normal honeycomb-like SECN and irregular collecting venules and areas of tubular structures in the gastric body mucosa (Figures 1B). Targeted biopsies from the gastric body showed glandular atrophy, intestinal metaplasia and inflammatory infiltrate in the gastric corpus, sparing the antrum, as in autoimmune gastritis (Figure 1C). On the other hand, the histological findings in the biopsied specimens from the antral mucosa showed no pathological changes. Histology showed no *Helicobacter pylori* micro-organisms. Serum anti-parietal cell antibodies were positive.

DISCUSSION

Autoimmune gastritis is characterized by autoimmune destruction of fundic and body glands. The marker for the most severe, end-stage form of diffuse corporal atrophic gastritis is pernicious anemia^[4]. However, no endoscopic findings specific for this entity have been reported.

The microvascular pattern of human gastric mucosa has recently been investigated^[5-7]. The normal gastric body and fundus mucosal microvessels show two major components: (1) subepithelial capillaries; and (2) collecting venules. Polygonal loops of subepithelial capillaries surround the neck of gastric pits. These loops form a honeycomb-like SECN and converge onto mucosal collecting

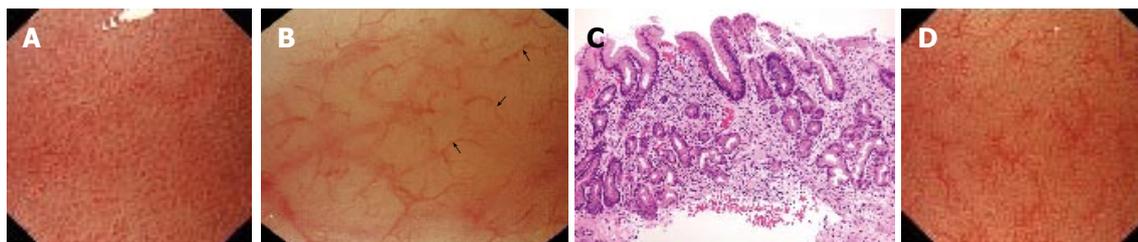


Figure 1 **A:** Magnified view of gastric antral mucosa in our patient. All parts of the antral mucosa demonstrated a coil-shaped subepithelial capillary network (SECN) in regular arrangement; **B:** Magnified endoscopic view of the gastric body mucosa in our patient. Loss of the normal SECN and collecting venules (arrows) in irregular shape and arrangement were evident. These findings are compatible with those of gastric atrophy; **C:** Histology reveals decreased density of glands, loss of specialised glands in the gastric body; **D:** Magnified view of normal gastric body mucosa in another healthy patient Honeycomb-like SECN with collecting venules in regular arrangement can be seen clearly.

venules that drain down into the submucosa (Figure 1D). In the antral mucosa, the normal subepithelial capillaries form a coil-shaped network, while collecting venules are rarely seen.

Yao *et al* have demonstrated that the resolution of the GIF-Q240Z gastroscope is 7.9 μm . Since the minimal diameter of subepithelial capillaries in the gastric mucosa, as described in anatomic studies, is 8 μm , this endoscope is ideally suited to visualize microvessels and capillaries^[6].

Nakagawa *et al* have recently studied the usefulness of magnifying endoscopy for the diagnosis of *H pylori*-induced histopathologic gastritis^[8]. The observed morphology of collecting venules was divided into the 3 patterns: (1) regular (R), which had the qualities of regularity in venules size, visible second or third order branches, and a uniform distance between venules; (2) irregular (I), which had the qualities of irregularity in size, inability to observe second or third order branches, and a lack of a uniform distance between the collecting venules, with venules sometimes fused to adjacent venules and sometimes lying horizontally; and (3) obscured (O), in which no collecting venules were visible. Observation of an R pattern indicates an absence of *H pylori* infection and histopathologic gastritis. Observation of an O or I pattern indicates the presence of histopathologic gastritis from *H pylori* infection, and an I pattern suggests the presence of gastric mucosal atrophy. We have also shown that in cases of gastric atrophy associated with *H pylori* infection, the normal honeycomb-like subepithelial capillary network (SECN) in gastric body disappears and the collecting venules become irregular^[9].

The magnified endoscopic findings of diffuse atrophy in gastric body mucosa and normal antrum associated with autoimmune gastritis have not yet been described. In our cases, the magnified views in gastric antrum revealed a normal coil-shaped subepithelial capillary network. On the other hand, in gastric body mucosa we demonstrated loss of the normal honeycomb-like SECN pattern with irregular collecting venules, suggestive of corporal gastric atrophy, as well as areas with tubulovillous structures as seen in intestinal metaplasia. These magnified endoscopic findings were constantly detected in all parts of the gastric corpus that were examined. These patterns are suggested to be

characteristic of autoimmune gastritis and were confirmed by histology.

In summary, it seems that irregular collecting venules can be recognised both in autoimmune- and *H pylori*-associated gastric atrophy. The dissemination of this finding can be used to help differentiate between these two types of gastritis. Gastric body predominant atrophy visualised by magnifying endoscopy, can be very useful in making a precise diagnosis of autoimmune gastritis.

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CASE REPORT

Coexistence of esophageal superficial carcinoma and multiple leiomyomas: A case report

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Abstract

Leiomyomas are the most common benign tumors of the esophagus. They usually occur as a single lesion or as two or three nodules. Only two cases of esophageal multiple leiomyomas comprising more than 10 nodules have been reported previously. Moreover, there have been few reports of esophageal squamous cell carcinoma overlying submucosal tumors. We describe a 71-year-old man who was diagnosed as having a superficial esophageal cancer coexisting with two or three leiomyoma nodules. During surgery, 10 or more nodules that had not been evident preoperatively were palpable in the submucosal and muscular layers throughout the esophagus. As intramural metastasis of the esophageal cancer was suspected, we considered additional lymphadenectomy, but had to rule out this option because of the patient's severe anoxemia. Microscopic examination revealed that all the nodules were leiomyomas (20 lesions, up to 3 cm in diameter), and that invasion of the carcinoma cells was limited to the submucosal layer overlying a relatively large leiomyoma. This is the first report of superficial esophageal cancer coexisting with numerous solitary leiomyomas. Multiple minute leiomyomas are often misdiagnosed as intramural metastasis, and a leiomyoma at the base of a carcinoma lesion can also be misdiagnosed as tumor invasion. The present case shows that accurate diagnosis is required for the management of patients with coexisting superficial esophageal cancer and multiple leiomyomas.

INTRODUCTION

Leiomyomas are the most common benign tumors of the esophagus, accounting for roughly two-thirds of all benign tumors of this organ. Although its overall incidence reported in autopsy ranges from 0.005% to 5%,^[1,2] this may be an underestimation because many small lesions are missed at autopsy unless the esophagus is examined carefully. Studies involving detailed histological examinations have reported the frequency to be higher, at 7.9%^[3]. Since esophageal leiomyomas are generally slow-growing and the size of tumors may not change for many years, most affected patients are asymptomatic. Considering the risk of surgery and the patient's quality of life, non-surgical management is recommended.

Surgical resection of esophageal leiomyomas is warranted only when: (1) there is severe dysphagia due to giant or multiple lesions, (2) there is a possibility of symptomatic transformation, (3) there is a need to obtain a definitive histological diagnosis, (4) malignancy can only be ruled out by surgical removal, or (5) malignant epithelial tumors are also present.

Both giant and multiple lesions involving the entire esophagus can cause severe and debilitating dysphagia. The presence of a giant lesion itself is a risk factor for malignancy, and such lesions should be treated surgically even if they cause no symptoms. Otherwise, esophageal leiomyoma usually appears as a solitary tumor (97%). Occasionally (less than 2.4% of all cases), more than one esophageal leiomyoma; i.e., generally two or three, may occur in the same patient. Multiple leiomyomas of the esophagus are extremely rare, and only two such cases, involving more than 10 nodules, have been reported

previously^{4,5}. Diagnosis of medium-sized leiomyomas is generally easy using endoscopic ultrasonography (EUS) and computerized tomography (CT). However, this is difficult when multiple leiomyomas coexist with carcinoma lesions. In cases where carcinoma overlies a submucosal leiomyoma, there is a possibility of overestimating the extent of tumor invasion, and multiple minute leiomyomas are sometimes misdiagnosed as intramural metastasis.

Here, we describe a case of superficial esophageal squamous cell carcinoma (SCC) coexisting with multiple (more than 20) leiomyoma lesions, and we discuss the management of such patients and the histogenesis of carcinomas overlying esophageal submucosal tumors (SMT).

CASE REPORT

The patient is a 71-year-old man who presented with a cervical tumor in October 2000. He was diagnosed as having a malignant lymphoma of the thyroid and esophageal leiomyomas. The thyroid tumor disappeared completely after chemotherapy, and the patient maintained a status of complete remission. Barium swallow esophagograms showed two smoothly rounded defects. The larger tumor, 40 mm in diameter and exhibiting two or three bumps, was located in the middle third of the esophagus. The smaller one, 20 mm in diameter, was in the lower third of the esophagus (Figure 1A). Both tumors were covered by normal mucosa, and showed no signs of malignancy in CT and EUS examinations. Therefore, we diagnosed these SMTs as two (or possibly three) leiomyomas. One year later, in December 2001, no marked changes were evident in a barium swallow examination (Figure 1B). However, endoscopy revealed a superficial ulcerative tumor (24 mm in diameter) in the lower third of the esophagus, overlying the smaller SMT (Figure 2: Left). Chromoendoscopy with Lugol's iodine solution demonstrated that the polypoid lesion was located within the non-staining area and that part of the surface was covered by normal mucosa (Figure 2: Right). Multiple biopsies revealed that the ulcerative tumor was SCC. EUS demonstrated that the cancer overlaid one of the leiomyomas, originating in the muscular layer, suggesting that the cancer may have invaded only as far as the submucosal layer. In February 2002, the patient underwent esophagectomy with a gastric pull-up reconstruction and cervical anastomosis via abdominal, left cervical and right thoracotomy incisions at the Department of Surgery I, Iwate Medical University School of Medicine, Morioka, Japan. During the operation, 10 or more nodules that had not been evident preoperatively were palpable in the submucosal and muscular layers throughout the esophagus. Intramural metastasis of the esophageal cancer was therefore suspected, and additional lymphadenectomy was considered. However, this option was ruled out because of the patient's severe anoxemia.

The resected specimen showed a superficial ulcerative tumor and multiple SMTs of various sizes (Figure 3). Macroscopically, about 10 SMTs were evident throughout the esophagus. Each SMT appeared as a well demarcated round nodule, and none of the tumors

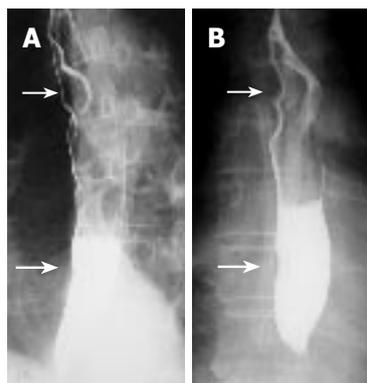


Figure 1 Barium swallow esophagograms. Lack of any marked change in two smooth round defects over the course of a year. **A:** On admission; **B:** One year later.

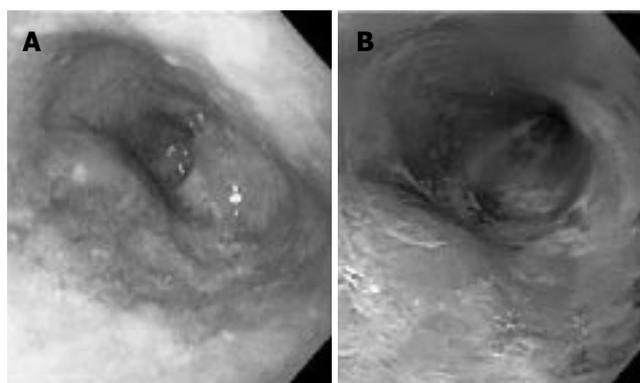


Figure 2 **A:** Endoscopic examination demonstrates a polypoid lesion with a partially irregular surface; **B:** Chromoendoscopy with Lugol's iodine solution demonstrates a polypoid lesion located within the non-staining area and normal mucosa covering part of the surface.

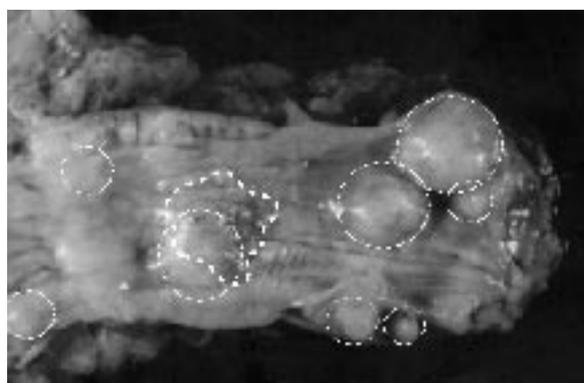


Figure 3 Resected specimen. Multiple polypoid tumors of various sizes are covered with normal mucosa (circles). A polypoid lesion in the lower third of the esophagus exhibits an irregular surface. The broken line indicates the area of SCC.

showed continuity with others, which is a characteristic feature of leiomyomatosis. Microscopically, more than 20 SMTs, measuring from 1 mm to 3 cm in diameter, were scattered throughout the esophagus (Figures 4 and 5: Arrows). All the SMTs showed low overall cellularity, and were composed of interlaced smooth-muscle cells with hypovascularity and no mitosis (Figure 5B). These findings are characteristic of leiomyoma. The ulcerative tumor overlaid one of the leiomyoma nodules in the lower third of the esophagus. The tumor was confirmed as SCC, and

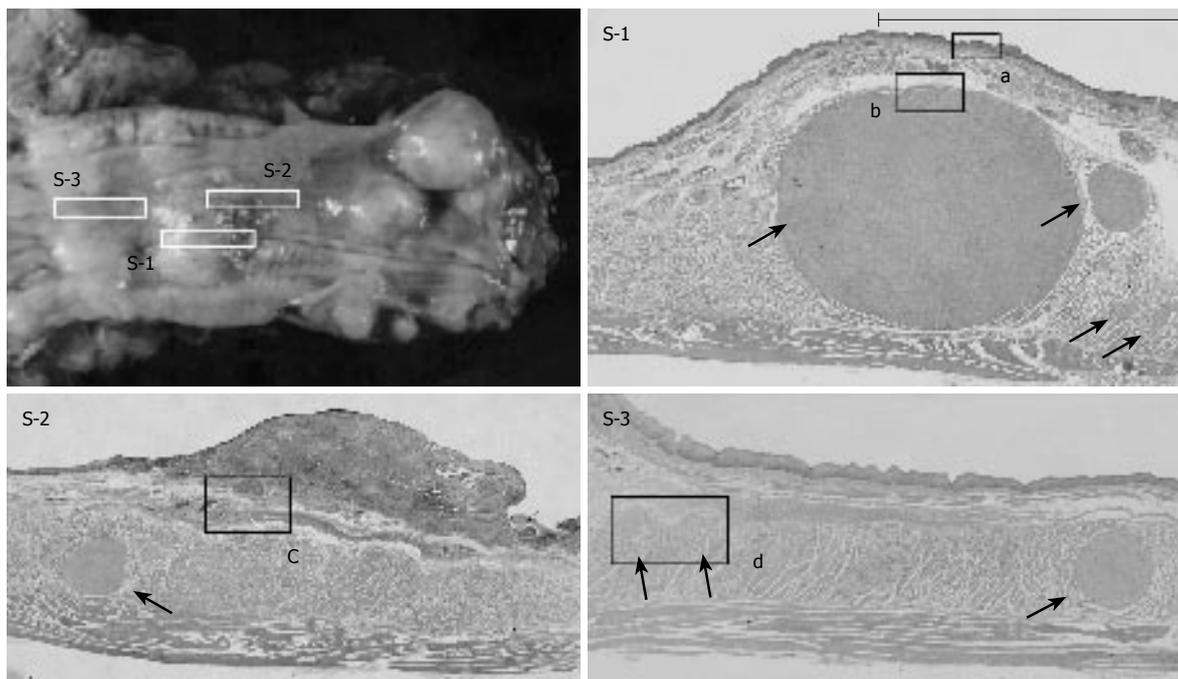
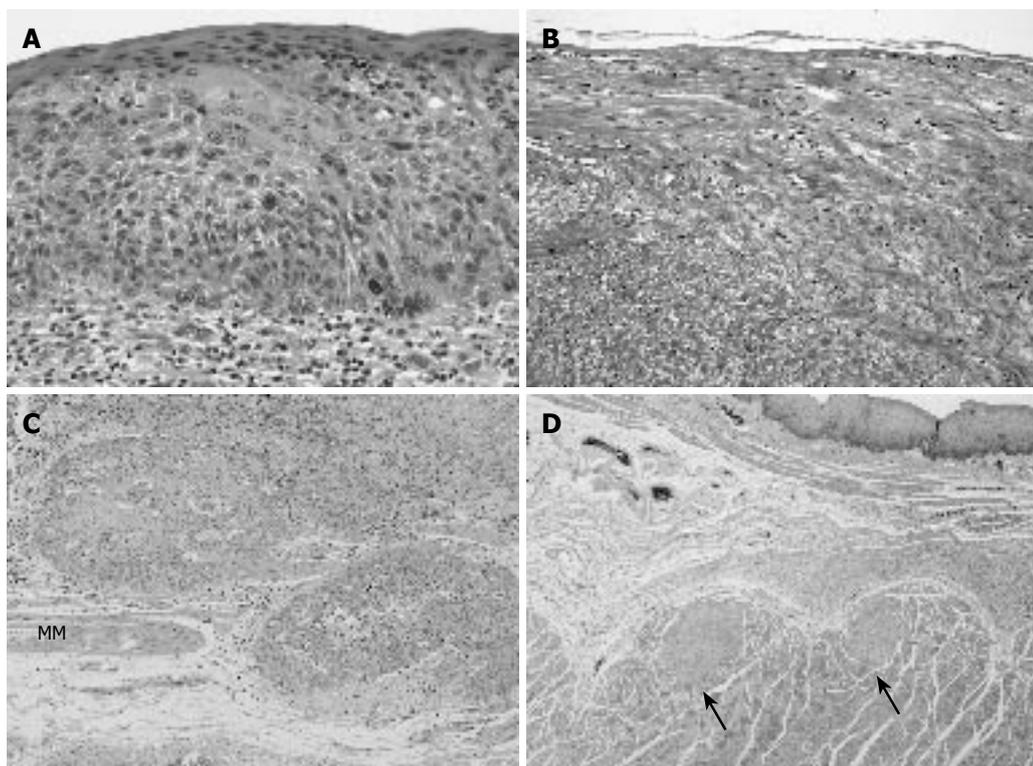


Figure 4 Photomicrograph of cross-sections (squares) of the resected specimen. Leiomyoma tissue is located in all sections (arrows). **S-1**: CIS overlies the leiomyoma. The region of CIS extends to the oral side of the leiomyoma (bar). **S-2**: SCC invades the submucosal layer in a very narrow region. **S-3**: A section without abnormal macroscopic findings. Multiple small leiomyomas are observed only by microscopic examination.



extensive carcinoma in situ (CIS) was observed on the leiomyoma (Figures 4 S-1, 5A). On the oral side of the SMT, the cancer had invaded into the submucosal layer, without lymph node metastasis (Figures 4 S-2, 5C).

The patient was discharged one month after the operation. At the time of writing, three years later, no local recurrence or distant metastasis of the SCC or malignant lymphoma has been observed.

DISCUSSION

As a distinct entity, multiple leiomyoma of the esophagus involving solitary lesions needs to be clearly distinguished from diffuse leiomyomatosis. Multiple leiomyoma of the esophagus is extremely rare, and only two such cases, involving more than 10 lesions, have been reported previously^[4,5]. Bradford *et al* reported a patient with 14

Table 1 Cases of ESC located in the surface epithelium over benign tumors

	Authors	Age	Sex	Submucosal tumor (SMT)	Size of SMT (cm)	Depth of cancer invasion	Subviews and <Management>
1	Marcial-Rojas RA	63	Male	Lipoma	11 × 3.5	Mucosal layer	<Cervical esophagotomy, excision>
2	Iizuka <i>et al</i>	75	Male	Leiomyoma	3.0 × 2.0	Mucosal layer	<Blunt dissection>
3	Watanabe <i>et al</i>	53	Male	Leiomyoma	0.8 × 0.6 × 0.25	Intraepithelium	Dysplasia <Chemotherapy, esophagectomy>
4	Callanan <i>et al</i>	54	Male	Leiomyoma	1.25	Muscular layer	<Esophagectomy>
5		45	Male	Leiomyoma	4	Muscular layer	<Esophagectomy>
6	Kuwano <i>et al</i>	63	Male	Leiomyoma	1	Mucosal layer	Dysplasia <Esophagectomy>
7		71	Male	Lipoma	1.5	Mucosal layer	<Esophagectomy>
8		52	Male	Leiomyoma	1.5 × 1.1 × 0.7	Mucosal layer	CIS and dysplasia <Esophagectomy>
9	Yosikane <i>et al</i>	67	Male	Lipoma	2.8 × 4.5	Intraepithelium	Separate cancer <Esophagectomy>
10	Nagashima <i>et al</i>	61	Male	Leiomyoma	2.9 × 2.0	Lamina propria	<Endoscopic resection>
11	Mizobuchi <i>et al</i>	64	Male	Leiomyoma	0.7 × 0.65	Lamina propria	<Esophagectomy>
12	Fu <i>et al</i>	62	Male	Leiomyoma	2	Mucosal layer	<Endoscopic resection>
13	Present case	73	Male	Leiomyoma	2.0 × 1.5	Submucosal layer	Multiple leiomyomas <Esophagectomy>

esophageal leiomyomas measuring 1 to 4 cm in diameter, and Seremetis *et al* reported another patient who had 15 such leiomyomas 0.4 to 6 cm in diameter. In our patient, more than 20 leiomyomas, measuring from 1 mm to 3 cm in diameter, were scattered throughout the esophagus.

There have been several reports of esophageal SCC coexisting with benign SMT^[6-14]. This presentation shows two types: one is the overlying type, where the carcinoma covers the benign SMT, and in the other type the two lesions are separate. Generally, most SMTs in the separate type are minute leiomyomas that are discovered only during postoperative examination of pathology specimens^[5]. This type of presentation is often encountered during esophagectomy for esophageal SCC. Otherwise, twelve cases of the overlying type have been reported previously^[6-14] and are summarized in Table 1. Although benign tumors of various sizes have been reported, all of them protruded into the esophageal lumen. Some investigators have speculated that the pathogenesis of the overlying type may involve chronic irritation of the esophageal mucosa, due to intraluminal protrusion of the SMT, leading to carcinoma development^[9-11]. This hypothesis has been suggested in reports of SCC coexisting with lipomas as well as leiomyomas^[15]. In the present patient, both overlying and separate types were observed. Although there were 20 leiomyomas scattered throughout the esophagus, the SCC overlaid a relatively large and protruding tumor. Thus, the size and location of SMTs might affect the degree to which the esophageal mucosa is exposed to chronic irritation.

Recently, curative endoscopic resection has been performed for patients with carcinomas overlying SMTs. However, such cases require accurate diagnosis of the depth of invasion using EUS^[7,8]. Mizobuchi *et al* reported a case in which an early esophageal SCC was overdiagnosed because the leiomyoma appeared to be a component of the carcinoma^[6]. In these reports, the authors emphasized that accurate diagnosis of the leiomyoma made it possible to avoid excessive surgery. Several diagnostic modalities, such as ballium swallow, endoscopy, CT and EUS, allow esophageal leiomyoma to be diagnosed effectively, and most of them have been

monitored^[16]. With these examinations, however, small leiomyomas are often difficult to diagnose. Indeed, as in the present case, a number of small SMTs may not be apparent preoperatively. During surgery, we were unable to completely rule out the possibility that some of the SMTs were intramural metastases of the ESC, as multiple leiomyoma of the esophagus is quite rare and intramural metastasis of esophageal superficial carcinoma is relatively common.

Since many coexisting carcinomas reported previously have been superficial, it has been speculated that the coexisting SMT may in fact inhibit the growth and invasion of the carcinoma^[10]. In the present case, the overlying SCC had invaded only as far as the mucosal layer, and submucosal invasion was limited to only a very narrow region where the SMT was absent. This case appears to support the speculation that SMT inhibits the growth of SCC. During the operation, lymphadenectomy was ruled out because of the patient's anoxemia. If conditions had been more favorable, the patient might have undergone more aggressive surgery. Therefore, in cases where a coexisting superficial carcinoma overlies a benign SMT, a more noninvasive therapeutic procedure (EMR, thoracoscopic approach, and omission of lymphadenectomy *etc.*) should be chosen.

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A case of gallbladder carcinoma associated with pancreatobiliary reflux in the absence of a pancreaticobiliary maljunction: A hint for early diagnosis of gallbladder carcinoma

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Abstract

A 62-year-old man with progressive thickening of the gallbladder wall visited our outpatient clinic. The biliary amylase level in the common bile duct was 19900 IU/L and that of the gallbladder was 127000 IU/L, although endoscopic retrograde cholangiopancreatography revealed no pancreaticobiliary maljunction. Histology demonstrated a moderately differentiated adenocarcinoma of the gallbladder. Pancreatobiliary reflux and associated gallbladder carcinoma were confirmed in the present case, in the absence of a pancreaticobiliary maljunction. Earlier detection of the pancreatobiliary reflux and progressive thickening of the gallbladder wall might have led to an earlier resection of the gallbladder and improved this patient's poor prognosis.

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Key words: Amylase; Bile; Gallbladder carcinoma; Pancreatobiliary reflux; Pancreaticobiliary maljunction; Diagnosis

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INTRODUCTION

Regurgitation of pancreatic juice into the biliary tract (pancreatobiliary reflux) usually occurs in patients with pancreaticobiliary maljunction and is closely related to the occurrence of biliary malignancy^[1,2]. Bile mixed with pancreatic

juice is known to induce cellular proliferation and stimulate genetic alterations of the biliary tract epithelium, leading to hyperplasia, dysplasia and ultimately, carcinoma of the biliary tract mucosa^[3].

Here we describe a patient with pancreatobiliary reflux in the absence of pancreaticobiliary maljunction, who showed progressive thickening of the gallbladder wall, which developed into an advanced gallbladder carcinoma.

CASE REPORT

A 62-year-old man visited our outpatient clinic for further evaluation of diffuse thickening of the gallbladder wall, detected by ultrasonography, at an annual medical check-up. He had no symptoms, and the physical examination showed no abnormalities. Laboratory test results were within normal limits. The serum levels of carcinoembryonic antigen was 1.3 ng/mL and the carbohydrate antigen 19-9 level was 1.0 U/mL; that is, both were within normal limits. Computed tomography (CT) revealed diffuse thickening of the gallbladder wall and dilatation of the common bile duct to 12 mm (Figure 1A). The patient was followed, and CT was scheduled for 3 mo later. He returned 42 mo later with persistent right hypochondrialgia and slight tenderness in the right upper quadrant. Laboratory tests, including serum amylase and lipase, were within the normal range. The serum levels of carcinoembryonic antigen was up to 2.1 ng/mL and the carbohydrate antigen 19-9 level increased to 5.0 U/mL, but both were still within the normal limits. CT revealed progressive thickening of the gallbladder wall and carcinoma of the gallbladder was suspected (Figure 1B). Endoscopic retrograde cholangiopancreatography (ERCP) revealed no pancreaticobiliary maljunction, because the length of the common channel was 5 mm, sphincter of Oddi affected the pancreaticobiliary junction and a connection between two the ducts was not visible during the contraction phase of sphincter of Oddi (Figure 2A, B). The biliary amylase level in the common bile duct, sampled during ERCP, was 19900 IU/L. Extended cholecystectomy, bile duct resection, and lymph node dissection were performed. The amylase level in gallbladder bile, sampled during cholecystectomy, was 127000 IU/L. Amylase was measured by an enzymatic method using the substrate 3-ketobutyliden-β-2-chloro-4-nitrophenyl-maltopentaoside (Diacolor Neonate; Toyobo, Osaka, Japan), and the normal range of serum amylase at our institution was 130-400 IU/L. The histopathologic di-

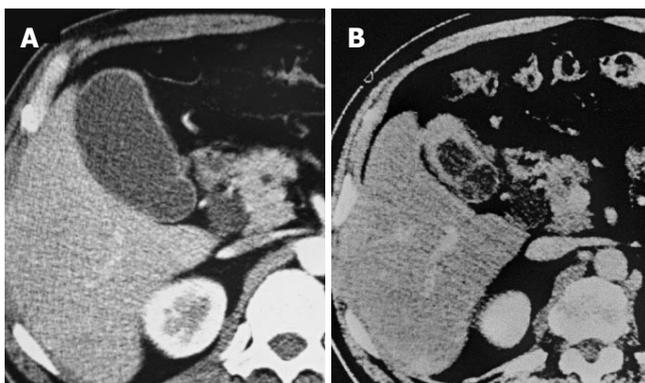


Figure 1 A: CT reveals diffuse thickening of the gallbladder wall and dilatation of the common bile duct to 14 mm; B: After 42 mo, CT reveals progressive thickening of the gallbladder wall, suspicious for gallbladder carcinoma.

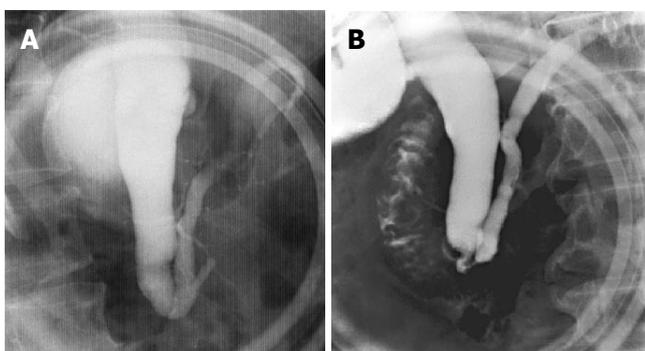


Figure 2 A ERCP shows no pancreaticobiliary maljunction, because sphincter of Oddi affected pancreaticobiliary junction and connection between pancreatic and biliary ducts was not visible during the contraction phase of sphincter of Oddi; B: ERCP in the relaxation phase of sphincter of Oddi shows the length of the common channel to be 5 mm long.

agnosis of the resected specimen was a moderately differentiated adenocarcinoma of the gallbladder's fundus with invasion of the serosa (Figure 3).

DISCUSSION

Pancreaticobiliary maljunction is defined as an abnormal union of the pancreatic and biliary ducts that is located outside the duodenal wall, where a sphincter system is not present^[1]. Thus, two ducts are always communicating, and pancreatic juice freely regurgitates into the biliary tract through this passage. Numerous clinical and experimental studies have supported a relationship between pancreaticobiliary reflux and biliary cancer in patients with pancreaticobiliary maljunction. Bile mixed with pancreatic juice is known to induce hyperplasia, metaplasia, and ultimately, carcinoma of the biliary tract mucosa in patients with pancreaticobiliary maljunction^[2,3]. In fact, biliary cancer is found in 15% to 67% of adult patients with pancreaticobiliary maljunction, and gallbladder carcinoma is found more often in patients with pancreaticobiliary maljunction without a congenital choledochal cyst^[4,5].

Pancreatobiliary reflux also occurred in our patient, in the absence of a pancreaticobiliary maljunction that was confirmed by elevated amylase levels in the bile sampled at

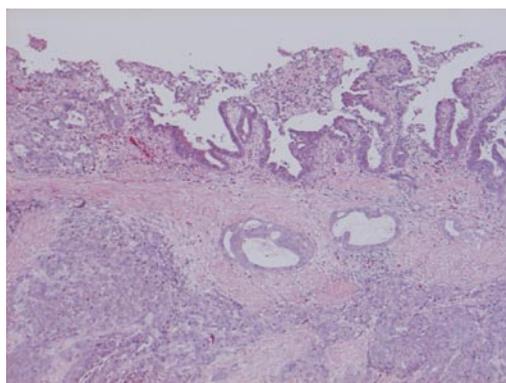


Figure 3 Histopathologic examination of the resected specimen demonstrates a moderately differentiated adenocarcinoma of the gallbladder (HE stain, $\times 40$).

ERCP and cholecystectomy. It was suspected that the extremely high levels of pancreatic enzymes in the bile might have induced carcinoma of the gallbladder as seen in patients with pancreaticobiliary maljunction^[4,5]. It was speculated that pancreatic juice may regurgitate into the biliary system through a common terminal ampulla that is known to exist in 60%-90% of human subjects^[6]; as pressure generated in the pancreatic duct is usually greater than that observed in the biliary system, it allows for flow from the pancreas to the biliary system^[7,8]. The precise mechanism of the pancreatobiliary reflux without pancreaticobiliary maljunction, and especially its relation to the dysfunction of sphincter of Oddi, should be further clarified.

One of the imaging findings that suggest mucosal changes accompanied by pancreatobiliary reflux is diffuse thickening of the gallbladder wall that might reflect cellular proliferation of the gallbladder epithelium, in patients with pancreaticobiliary maljunction^[2]. In the present case, diffuse thickening of the gallbladder wall was confirmed on ultrasonography and CT, and its relation to pancreatobiliary reflux could be suggested.

In the present case, pancreatobiliary reflux and associated gallbladder carcinoma were confirmed, in the absence of pancreaticobiliary maljunction. Earlier detection of the pancreatobiliary reflux and progressive thickening of the gallbladder wall might have led to earlier resection of the gallbladder and improved this patient's poor prognosis.

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CASE REPORT

A case of peribiliary cysts accompanying bile duct carcinoma

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Abstract

A rare case of peribiliary cysts accompanying bile duct carcinoma is presented. A 54-year-old man was diagnosed as having lower bile duct carcinoma and peribiliary cysts by diagnostic imaging. He underwent pylorus preserving pancreaticoduodenectomy. As for the peribiliary cysts, a course of observation was taken. Over surgery due to misdiagnosis of patients with biliary malignancy accompanied by peribiliary cysts should be avoided.

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Key words: Peribiliary cysts; Bile duct carcinoma; Intrahepatic cholangiocarcinoma

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INTRODUCTION

Hepatic peribiliary cysts are a poorly recognized liver disease characterized by multiple tiny cysts along the portal radicle. This type of lesion is derived from the cystic dilatation of intrahepatic extramural peribiliary glands around the intrahepatic large bile ducts^[1]. Although peribiliary cysts sometimes cause stricture of the intrahepatic bile duct and mimics intrahepatic cholangiocarcinoma^[2], to our knowledge there are no reports of this condition accompanying bile duct carcinoma. We report a rare case of peribiliary cysts accompanying bile duct carcinoma.

CASE REPORT

A 54-year-old Japanese man was admitted to a local hospital for complaints of jaundice. He was diagnosed with lower bile duct carcinoma and referred to our hospital after placement of endoscopic naso-biliary drainage. He had a history of heavy alcohol use and had been hospitalized for one year with alcoholism ten years previously. Physical examination results were generally unremarkable. The liver was palpable two fingerbreadths below the right costal margin. No ascites or edema was present. Laboratory examination revealed a normal complete blood count and normal coagulation. Liver function test results at admission to our hospital were as follows: aspartate aminotransferase (AST), 75 U/I; alanine aminotransferase (ALT), 133 U/I; alkaline phosphatase (ALP), 467 U; γ -glutamyl transpeptidase (GTP), 196 U/I; total bilirubin, 1.1 mg/mL; indocyanine green test (at 15 min), 6.2%. Hepatitis virus screening was negative and no tumor markers were elevated. Abdominal ultrasonography revealed multiple small cystic lesions and tubular anechoic areas along the umbilical portion of the portal vein that were difficult to differentiate with dilatation of the intrahepatic bile ducts. CT revealed a tumor in the lower bile duct and multiple small cystic lesions along the umbilical portion of the portal vein (Figure 1). Cholangiography *via* an endoscopic naso-biliary drainage tube revealed complete obstruction of the lower bile duct, smooth stenosis of the left hepatic duct, and a slight dilatation of the intrahepatic bile duct in the left lobe (Figure 2). Magnetic resonance cholangiopancreatography (MRCP) showed bead-like cystic lesions along the biliary tree in the left lobe of the liver (Figure 3). The left hepatic duct stenosis appeared to be caused by extramural compression by these cysts. Portal phase superior mesenteric arteriography and hepatic arteriography demonstrated no particular abnormalities. Based on these imaging studies, he was diagnosed as having lower bile duct carcinoma and peribiliary cysts. He underwent pylorus preserving pancreaticoduodenectomy. As for the peribiliary cysts, a course of observation was decided upon. The carcinoma was a nodal infiltrating-type tumor sized 12 mm \times 8 mm existing in the lower bile duct.

The tumor was histopathologically diagnosed as a moderately differentiated tubular adenocarcinoma, pT3, pN0, pM0 and stage IIA^[3]. After the operation, minor leakage of the pancreatojejunostomy developed and was successfully treated by conservative therapy. He is alive without recurrence and symptoms 10 mo after surgery at this writing.

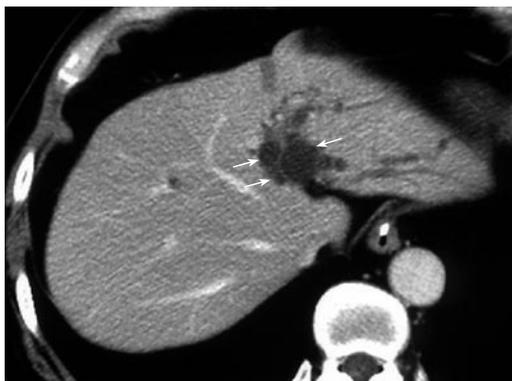


Figure 1 CT revealing multiple small cystic lesions along the umbilical portion of the portal vein (arrows).



Figure 2 Cholangiogram via endoscopic naso-biliary drainage tube revealing complete obstruction in the lower bile duct (black arrow) and smooth stenosis of the left hepatic duct (white arrows) and a slight dilatation of the intrahepatic bile duct in the left lobe.

DISCUSSION

Peribiliary cysts were first reported by Nakanuma *et al* in 1984^[4]. They speculated that the peribiliary cysts arose from cystic dilatation of the intrahepatic extramural peribiliary glands in the periductal connective tissue^[4]. A systematic study of autopsied livers disclosed that the disease is not uncommon; cystic changes of the peribiliary glands were found in 202 livers (20.2%) among 1000 consecutive autopsy livers^[1]. However, since the degree of cystic dilatation is quite variable, and the majority are recognizable microscopically, this disease is still poorly recognized in clinical practice^[5]. The precise mechanism for cystic dilatations of intrahepatic peribiliary glands is unclear. Peribiliary cysts are usually detectable in pre-existing hepatobiliary diseases such as liver cirrhosis, idiopathic portal hypertension, portal vein thrombosis, alcoholic liver disease, and adult-type polycystic kidney disease^[4,6,7]. Therefore, it has been speculated that circulation failure caused by the interference of blood flow through the portal vein causes morphological changes of extramural glands around the bile duct. Nakanuma presumed that, in cases of adult-type polycystic kidney disease, gene expression in the intrahepatic peribiliary glands related to this disease may lead to the formation of peribiliary cysts^[5]. In the case reported here, alcoholic liver injury did not develop into liver cirrhosis, and there were no findings of portal hypertension. Peribiliary cysts are also known to develop in patients with cystic dilatation of the intrahepatic biliary system after hepatic portoenterostomy^[8]. We speculate that in the case reported here, cholestasia due to lower bile duct cancer, in addition to alcoholic liver disease, might be related to the development of peribiliary cysts. However, whether peribiliary cysts existed before the development of bile duct cancer is uncertain, since he had not undergone diagnostic imaging before the onset of obstructive jaundice.

On diagnostic imaging, peribiliary cysts are characterized as clusters of relatively small multiple cystic lesions developing around the hepatic hilum and large branches of the portal vein, but not in liver parenchyma^[9]. Recently, MRCP has been reported to be useful in the diagnosis of peribiliary cysts. MRCP clearly depicts

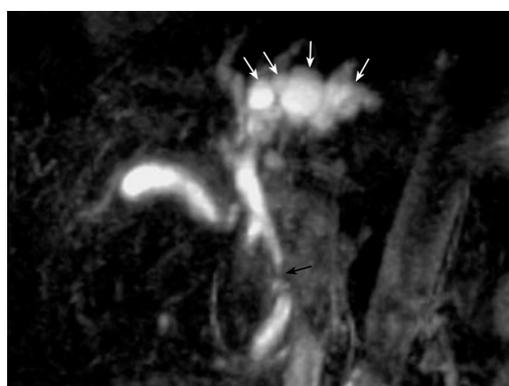


Figure 3 Magnetic resonance cholangiopancreatogram showing bead-like cystic lesions (arrows) along the biliary tree in the left lobe of the liver.

multiple cysts as a string of bead-like structures or a foamy fringe along the hepatic hilum or larger bile ducts. Drip infusion cholangiographic (DIC) CT is also useful for diagnosing peribiliary cysts. On DIC-CT, peribiliary cysts are not filled with contrast material since these cysts do not communicate with the lumen of the biliary tree.

Peribiliary cysts have usually been considered to be clinically harmless. There are two reports describing obstructive jaundice secondary to the obstruction of bile ducts by peribiliary cysts^[2,10]. It is thought that surgery is not indicated for asymptomatic peribiliary cysts but they should be followed up^[9,11]. Misdiagnosis of peribiliary cysts as intrahepatic cholangiocarcinoma is a possibility^[2,12,13], and there are two reports concerning such patients undergoing hepatectomy on suspicion of intrahepatic cholangiocarcinoma^[12,13]. Precise diagnosis can be obtained easily by characteristic findings with diagnostic imaging if the attending clinician is knowledgeable about peribiliary cysts. Needless to say, unnecessary operations for patients with peribiliary cysts should be avoided.

There have been no reports of peribiliary cysts accompanying bile duct carcinoma. If knowledge of the clinical entity of peribiliary cysts becomes widespread, the number of patients with peribiliary cysts accompanying biliary malignancy is sure to increase. When peribiliary

cysts and biliary malignancy exist simultaneously, the operative procedure should be chosen according to the staging and extension of the malignancy as well as the positional relationship between the two lesions based on proper diagnosis. We chose not to select surgical therapy for the peribiliary cysts, on the basis that we diagnosed the stricture of the left hepatic duct as compression by peribiliary cysts, and hepatopancreatoduodenectomy, an indispensable procedure for resecting both the lower bile duct cancer and stricture of left hepatic duct, by itself is associated with high rates of postoperative complications and operative mortality^[14]. Over-surgery due to misdiagnosis for patients with biliary malignancy accompanied by peribiliary cysts should be avoided. However, peribiliary cysts have been reported to become gradually enlarged and increased in number in some cases^[15,16]. Long-term follow-up is obviously called for.

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Volvulus of the gall bladder diagnosed by ultrasonography, computed tomography, coronal magnetic resonance imaging and magnetic resonance cholangio-pancreatography

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Abstract

A 54-year-old woman was admitted to our hospital with the complaint of right upper quadrant pain. Upon physical examination the vital signs of the patient were within normal ranges. Ultrasonography and computed tomography (CT) examination of the abdomen was obtained, which demonstrated a large dilated cystic structure, measuring approximately 68.6 mm × 48.6 mm, with marked distension and inflammation. Additionally, the enhanced CT was characterized by the non-enhanced wall of the gallbladder. As the third examination in this study, magnetic resonance imaging (MRI), namely coronal MRI and magnetic resonance cholangio-pancreatography (MRCP), were performed. The MRCP demonstrated a dilatation of the gallbladder but detected no neck of the gallbladder. Simple cholecystectomy was performed. Macroscopic findings included a distended and gangrenous gallbladder, and closer examination revealed a counterclockwise torsion of 360 degrees on the gallbladder mesentery. Coronal MRI and MRCP showing characteristic radiography may be useful in making a definitive diagnosis.

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Key words: Volvulus of the gallbladder; Computed tomography; Magnetic resonance imaging; Magnetic

resonance cholangio-pancreatography

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INTRODUCTION

Gallbladder volvulus is a relatively rare disease and well recognized in the elderly people. It has been reported in only about 300 cases in the literature ranging in age from 2 to 100 years old. Preoperative diagnosis of gallbladder volvulus has always been considered difficult. Although recent advances in radiographic finding have helped in the diagnosis of many diseases, abdominal computed tomography (CT) and Ultrasonography (US) remain nonspecific in diagnosing volvulus of the gallbladder. However, we could make definitive radiographic imaging by coronal magnetic resonance imaging (MRI) and magnetic resonance cholangio-pancreatography (MRCP).

CASE REPORT

A 54-year-old woman was admitted to our hospital with the complaint of right upper quadrant pain of approximately 5 hours in duration. Pain was accompanied by anorexia and nausea without vomiting and was not preceded by jaundice. She had no relevant past history. Upon physical examination the vital signs of the patient were within normal ranges. Right upper quadrant tenderness and Murphy's sign were detected in the abdominal examination. Laboratory data showed a leukocyte count of 11 800/mm³ and C-reactive protein (CRP) of 2.57 mg/dL. Initial treatment consisted of administering intravenous fluids and broad-spectrum antibiotics. Abdominal ultrasound US demonstrated a distended, fluid-filled neck of abnormal swelling, a normal-walled gallbladder with surrounding ascites and edema (Figure 1), but no stones. Secondary



Figure 1 Abdominal ultrasonography revealed an abnormally large floating gallbladder without gallstones, and a thickened gallbladder wall.



Figure 2 Abdominal enhanced computed tomography revealed a dilated gallbladder, but a non-enhanced gallbladder wall.

computed tomography (CT) examination of the abdomen was obtained. CT demonstrated a large dilated cystic structure, measuring approximately 68.6 mm × 48.6 mm, with marked distension and inflammation. Additionally, the enhanced CT was characterized by the non-enhanced wall of the gallbladder (Figure 2). As the third examination in this study, magnetic resonance imaging (MRI), namely coronal MRI and magnetic resonance cholangiopancreatography (MRCP), were performed. The MRCP demonstrated a dilatation of the gallbladder but detected no neck of the gallbladder (Figure 3). The coronal MRI revealed a dilated gallbladder, and additionally an invagination that identified the neck of the gallbladder (Figure 4). She was diagnosed with acute cholecystitis and volvulus of the gallbladder according to the findings of US, CT, MRI and MRCP and underwent percutaneous transhepatic gallbladder aspiration (PTGBA) for one day. Following the treatment, a hemobilia discharge from the PTGBA was noted, and she was diagnosed with necrotic gallbladder. Her pain was slightly improved, but she still had tenderness and high fever. She underwent open laparotomy, and simple cholecystectomy was performed. Macroscopic findings included a distended and gangrenous gallbladder, and closer examination revealed a counterclockwise torsion of 360 degrees on the gallbladder mesentery. De-torsion and cholecystectomy were easily performed (Figure 5). The histopathology report showed

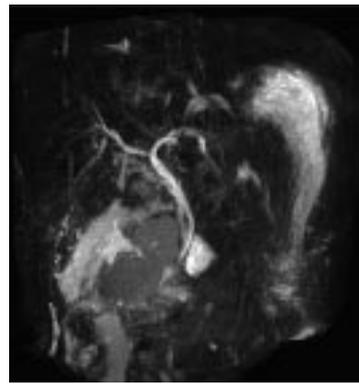


Figure 3 MRCP demonstrated dilatation of the gallbladder, but its image identified no gallbladder neck.



Figure 4 Coronal MRI revealed a dilated gallbladder, and its invagination-like image identified the neck of the gallbladder.



Figure 5 At laparotomy, macroscopic findings showed a distended and gangrenous gallbladder along with a counterclockwise torsion of 360 degrees of the gallbladder mesentery.

necrosis and hemorrhage of the gallbladder without evidence of lithiasis. Nine days after the operation she was discharged with no complications.

DISCUSSION

First reported by Wendel in 1898^[1], volvulus of the gallbladder is a relatively uncommon phenomenon, with no more than 300 cases reported in the literature. It occurs in all age groups, with the highest incidence in elderly women, and a female-to-male ratio of 3:1. Perhaps the incidence would increase with a longer life expectancy rate^[2,3]. Gallstones are unlikely to be the cause of gallbladder torsion, as gallstones are not uniformly present in all patients reported with torsion. One study of

245 patients found stones in only 24.4%; 51% developed a clockwise torsion rotation^[4]. Although supportive evidence is lacking, inferences have been made in the literature linking gastric peristalsis to clockwise gallbladder torsion and colonic peristalsis to counterclockwise torsion^[5]. Because volvulus of the gallbladder is a relatively uncommon phenomenon, preoperative diagnosis of gallbladder torsion remains difficult. Therefore, most cases are diagnosed intraoperatively at present. Also, laboratory evaluations are often nonspecific. For example, white blood cell count (WBC), CRP and creatine phosphokinase (CPK) are frequently elevated, while liver function tests are commonly normal. In our case, WBC, CRP and CPK were elevated. Although recent advances in radiographic studies have helped in the diagnosis of many diseases, radiographic studies remain nonspecific in diagnosing volvulus of the gallbladder. Fewer than a dozen cases have been reported in the literature where a preoperative diagnosis was made. Ultrasound studies often reveal a large floating gallbladder without gallstones, and a thickened gallbladder wall. Specific ultrasound signs seen with gallbladder torsion include the presence of the gallbladder outside its normal anatomic fossa, inferior to the liver or in a transverse orientation with an echogenic conical structure^[6]. Additionally, CT findings are also nonspecific. A few cases of CT diagnosis of gallbladder torsion commented on radiographic findings of marked enlargement of the gallbladder with an unusual shape and configuration^[7,8]. MRI has been used to establish a diagnosis preoperatively. MRI findings include high signal intensity within the gallbladder wall on T1 weighted images, suggesting necrosis and hemorrhage consistent with gallbladder torsion. In the present case, MRCP revealed anatomic details of the neck of the gallbladder and cystic duct.

We consider the characteristics of these radiography images to be useful for differential diagnosis of torsion of the gallbladder from gallbladder stone and gallbladder cancer. Usui *et al* have reported that only MRCP made it possible to determine the relationships between the distorted bile ducts, the interrupted cystic duct, and the enlarged gallbladder, and it was a relatively non-invasive procedure^[9]. In addition, Shaikh *et al* have reported that the presence of a redundant mesentry was a prerequisite

for torsion^[10]. Although torsion of the gallbladder is a rare occurrence, the diagnosis should be considered in all patients presenting with right upper quadrant pain. If diagnosed early and treated, it remains a benign condition; however, a delay in diagnosis and management may lead to sequelae associated with gallbladder rupture and biliary peritonitis. US, CT, and magnetic resonance techniques, especially coronal MRI and MRCP, are useful in diagnosing volvulus of the gallbladder. About 300 cases have been described in the literature so far, but only a minor portion (putatively less than 50 cases) have preoperative imaging studies such as CT and MRI. Ours is the first case in the medical literature in English to report on volvulus of the gallbladder diagnosed by coronal MRI and MRCP showing characteristic radiography.

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CASE REPORT

Branch retinal vein thrombosis and visual loss probably associated with pegylated interferon therapy of chronic hepatitis C

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Abstract

Ophthalmological complications with interferon therapy are usually mild and reversible, not requiring the withdrawal of the treatment. We report a case of a patient who had visual loss probably associated with interferon therapy. Chronic hepatitis C virus infection (genotype 1a) was diagnosed in a 33-year old asymptomatic man. His past medical history was unremarkable and previous routine ophthalmologic check-up was normal. Pegylated interferon alpha and ribavirin were started. Three weeks later he reported painless reduction of vision. Ophthalmologic examination showed extensive intraretinal hemorrhages and cotton-wool spots, associated with inferior branch retinal vein thrombosis. Antiviral therapy was immediately discontinued, but one year later he persists with severely decreased visual acuity. This case illustrates the possibility of unpredictable and severe complications during pegylated interferon therapy.

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Key words: Hepatitis C; Interferon; Visual loss

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<http://www.wjgnet.com/1007-9327/12/4602.asp>

INTRODUCTION

Pegylated interferon has been shown to be associated with high rates of sustained virological responses and has become the standard therapy of chronic hepatitis C. However, interferon therapy may be associated with severe adverse effects. We report the case of a patient who developed retinal vein thrombosis and visual loss probably related to the antiviral therapy.

CASE REPORT

A 33-year old asymptomatic Caucasian man was referred for investigation of abnormal liver enzymes. Chronic hepatitis C virus infection, genotype 1a, with viral load 220 400 IU/mL, was diagnosed, and a liver biopsy showed chronic hepatitis, with inflammatory activity A2 and fibrosis F2 according to the METAVIR system. Past medical history was unremarkable and negative for diabetes mellitus, arterial hypertension, dislipidemia, obesity, regular use of medications, smoking and illicit drug or alcohol abuse. Previous routine ophthalmologic check-up was normal, with a visual acuity 20/20. Pegylated interferon α 2b was started at the dose of 1.5 μ g/kg once weekly in combination with ribavirin 1000 mg/d. Three weeks later he reported painless blurring of vision. The ophthalmologic examination confirmed the reduction of the visual acuity and showed extensive intraretinal hemorrhages and cotton-wool spots, associated with inferior branch retinal vein thrombosis. Fluorescein angiography confirmed the venous occlusion (Figure 1). The antiviral therapy was immediately discontinued. Hematocrit, platelet count, glucose, prothrombin activity, creatinine, cholesterol and triglycerides levels were normal. Activated protein C resistance phenotype was normal. The levels of protein S, antithrombin III, homocysteine and fibrinogen were within the normal ranges. Plasma protein C was 50% (normal 58%-125%), compatible with heterozygous state. Cryoglobulins and antiphospholipids antibodies were negative. Factor V Q 506 and prothrombin gene 3'-UTR G20210A mutant alleles were absent. Doppler ultrasound of the heart and carotid arteries was normal. The patient developed macular edema which required panretinal photocoagulation. One year later, he persists with severely decreased visual acuity.

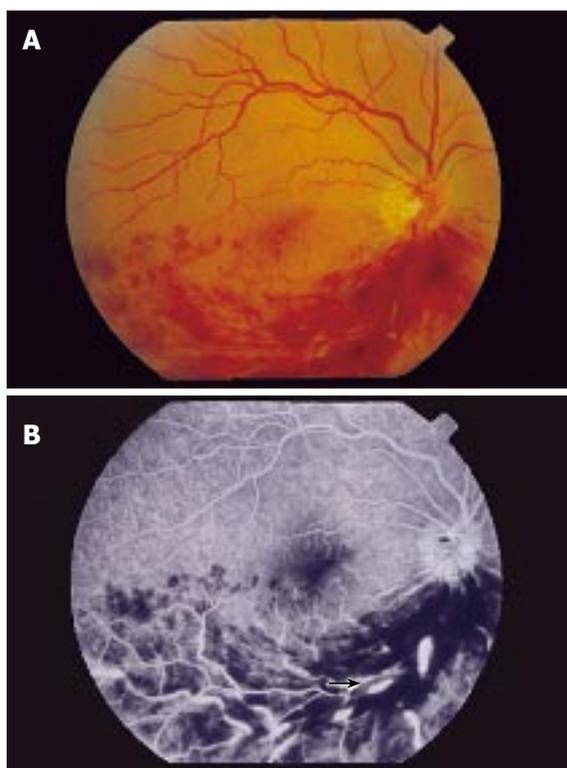


Figure 1 **A:** Fundus photograph of the right eye with extensive intraretinal hemorrhages and cotton-wool spots in the inferonasal and inferotemporal regions; **B:** Fluorescein angiography with segmental hypoperfusion, dilation and tortuosity of the retinal veins (arrow), compatible with inferior branch retinal vein thrombosis.

DISCUSSION

Ophthalmologic complications with interferon alpha therapy, such as retinopathy with cotton-wool spots, hemorrhages and macular edema, optic neuropathy and thrombotic microangiopathy, occur in less than 1% of treated patients. Individuals with diabetes, hypertension, dyslipidemia and hypercoagulable states are more prone to develop those changes. In most cases they are subclinical, mild and reversible, not requiring the withdrawal of the treatment.

The physiopathology of retinal vein thrombosis in patients with viral hepatitis is poorly understood, but may be related to both background predisposition and side effects of therapy. Protein C deficiency occurs in 1 of 250

controls and leads to impaired inhibition of clot formation, which is associated with an increased risk for venous thrombosis (relative risk, 7.3; annual incidence, 1%)^[1]. However, the frequency of venous thrombosis in protein C deficiency state is highly variable. Only a minority of affected individuals develop thrombosis, suggesting that the presence of another simultaneous risk factor is needed.

Both chronic hepatitis C and interferon therapy are associated with the formation of procoagulant antibodies, in particular antiphospholipids antibodies, which may predispose to thrombosis. In addition, interferon therapy induces the increase of plasma-activated complement C5a, a potent intravascular aggregator of granulocytes, favoring the development of microthrombi in small vessels^[2,3].

The patient developed a severe, early and sight losing complication of antiviral therapy. Most reported cases occurred in predisposed individuals 2 to 11 months after the beginning of standard alpha interferon therapy. However, one could speculate that interferon pegylation could modify both the pharmacokinetics of the drug and the timing of retinal vein thrombosis.

Since there are few reported cases of retinal vein thrombosis^[3-5], this case may represent a coincidence, not associated with interferon therapy. Nevertheless, it is noteworthy that the patient was asymptomatic and had a normal vision before antiviral therapy. Although current guidelines of therapy of chronic viral hepatitis do not support routine ophthalmologic screening and work-up for hypercoagulable states in all patients treated with interferon, this case illustrates the possibility of unpredictable and severe complications during pegylated interferon therapy.

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Meetings

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First Biennial Congress of the Asian-Pacific Hepato-Pancreato-Biliary Association March, 2007
Fukuoka, Japan
<http://www.congre.co.jp/1st-aphba>

American College of Gastroenterology Annual Scientific
20-25 October 2006
Las Vegas, NV

14th United European Gastroenterology Week, UEGW
21-25 October 2006
Berlin, Germany

APDW 2006: Asian Pacific Digestive Week 2006
26-29 November 2006
Lahug Cebu City, Philippines

EVENTS AND MEETINGS IN THE UPCOMING 6 MONTHS

Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
24-25 March 2006
Sydney - NSW
Falk Foundation e.V.
symposia@falkfoundation.de

10th International Congress of Obesity
3-8 September 2006
Sydney
Event Planners Australia
enquiries@ico2006.com
www.ico2006.com

Easl 2006 - the 41st annual
26-30 April 2006
Vienna, Austria
Kenes International

Prague hepatology 2006
14-16 September 2006
Prague
Foundation of the Czech Society of Hepatology
veronika.revicka@congressprague.cz
www.czech-hepatology.cz/phm2006

12th International Symposium on Viral Hepatitis and Liver Disease
1-5 July 2006
Paris
MCI France
isvhld2006@mci-group.com
www.isvhld2006.com

Falk Symposium 152: Intestinal Disease Part I, Endoscopy 2006 - Update and Live Demonstration
4-5 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

Falk Symposium 153: Intestinal Disease Part II, Immunoregulation in Inflammatory Bowel Disease - Current Understanding and Innovation
6-7 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

ILTS 12th Annual International Congress
3-6 May 2006
Milan
ILTS
www.its.org

Internal Medicine: Gastroenterology
22 July 2006-1 August 2006
Amsterdam
Continuing Education Inc
jbarnhart@continuingeducation.net
6th Annual Gastroenterology And

Hepatology
15-18 March 2006
Rio Grande
Office of Continuing Medical Education
cmenet@jhmi.edu
www.hopkinscme.net

World Congress on Gastrointestinal Cancer
28 June 2006-1 July 2006
Barcelona, Spain
c.chase@imedex.com

International Conference on Surgical Infections, ICSI2006
6-8 September 2006
Stockholm
European Society of Clinical Microbiology and Infectious Diseases
icsi2006@stocon.se
www.icsi2006.se/9/23312.asp

7th World Congress of the International Hepato-Pancreato-Biliary Association
3-7 September 2006
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convention@edinburgh.org
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Society of American Gastrointestinal Endoscopic Surgeons
26-29 April 2006
Dallas - TX
www.sages.org

Digestive Disease Week 2006
20-25 May 2006
Los Angeles
www.ddw.org

Annual Postgraduate Course
25-26 May 2006
Los Angeles, CA
American Society of Gastrointestinal Endoscopy
www.asge.org/education

American Society of Colon and Rectal Surgeons
3-7 June 2006
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www.fascrs.org

EVENTS AND MEETINGS IN 2006

10th World Congress of the International Society for Diseases of the Esophagus
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- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci U S A* 2006; In press

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Issue with no volume

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- 9 Outreach: bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/EID/eid.htm>

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Slow transit constipation: A functional disorder becomes an enteric neuropathy

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Abstract

Slow transit constipation has been traditionally considered and classified as a functional disorder. However, clinical and manometric evidence has been accumulating that suggests how most of the motility alterations in STC might be considered of neuropathic type. In addition, further investigations showed that subtle alterations of the enteric nervous system, not evident to conventional histological examination, may be present in these patients. In the present article we will discuss these evidences, and will try to put them in relation with the abnormal motor function of the large bowel documented in this pathological condition.

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INTRODUCTION

In gastroenterology practice, the most frequently encountered disorders are represented by those related to an abnormal function of the abdominal viscera, the so-called functional diseases^[1,2]. The term “functional” defines several variable combinations of chronic or recurrent gastrointestinal symptoms that do not have an identified underlying pathophysiology^[3], and that are often also labelled as “idiopathic”. Thus, the definition of functional relies on the exclusion of an organic disease, and it is

consequently thought to be related to abnormalities of its physiological properties or functions.

Among the so-called functional disorders, one of the most common is functional constipation, whose diagnostic criteria according to Rome II classification^[4] are reported in Table 1. Patients with functional constipation may be further classified in three subgroups: normal transit constipation, disorders of defecatory or rectal evacuation (outlet obstruction), and slow transit constipation (STC)^[5,6]. This latter condition affects mainly women, it is characterized by an often intractable constipation, a heavily delayed colonic transit up to true colonic inertia^[7,8], and it is usually attributed to disorders of colonic motor function^[9,10]. Indeed, several abnormal motor aspects have been described in STC, such as alterations of rectosigmoid contractile activity^[11], decreased colonic propulsive function^[12,13], abnormal response to food ingestion^[14,15], and overall reduced electrical or motor activity of the large bowel^[16,17]. It must also be taken into account that a further cause of delayed colonic transit may be due to the association with pelvic floor dyssynergia; this condition must be identified, since it is amenable of biofeedback treatment, which can normalize colonic transit^[18].

However, we still do not know how and why these abnormalities are present in STC patients. In recent years, clinical and manometric evidence has been accumulated that suggests how most of the motility alterations in STC might be considered as a neuropathic type. Moreover, other data showed that some subtle alterations of the enteric nervous system, not evident to conventional histological examination, may be present in these patients. In the present article we will discuss these evidences, and will try to put them in relation with the abnormal motor function of the large bowel documented in this pathological condition.

STC AS A NEUROPATHY: CLINICAL EVIDENCE

In several patients with STC subclinical features of autonomic neuropathy may be present^[19], and other studies described selective sensory and autonomic neuropathy in these subjects^[20], often with a positive family history, suggesting a genetic basis for this condition^[21]. Animal studies might help in elucidating these issues: for instance, transgenic mice with a targeted deletion of neurturin (a neurotrophin) display clinical and tissue phenotype similar to that found in STC, and display associated

Table 1 Rome II criteria for functional constipation (adapted from reference 4)

Two or more of the following for at least 12 wk (not necessarily consecutive) in the preceding 12 mo:

- Straining during > 25% of bowel movements;
 - Lumpy/hard stools for > 25% of bowel movements;
 - Sensation of incomplete evacuation for > 25% of bowel movements;
 - Sensation of anorectal blockage for > 25% of bowel movements;
 - Manual manoeuvres to facilitate > 25% of bowel movements (e.g., digital evacuation or support of the pelvic floor);
 - < 3 bowel movements per week
- Loose stools are not present, and there are insufficient criteria for irritable bowel syndrome

parasympathetic deficits^[22].

Moreover, since in a sizable proportion of STC patients the symptoms start after pelvic surgical procedures^[23-25] or following childbirth^[26] it has been hypothesized (although the anatomic proof has never been given) that pelvic nerve injury may occur following hysterectomy and childbirth, and that STC could be considered a disorder of pelvic autonomic nerves at least in a subgroup of patients^[27].

STC AS AN ENTERIC NEUROPATHY: INSTRUMENTAL EVIDENCE

Several manometric studies carried out in patients with STC have reported findings that suggest the presence of neuropathic-type abnormalities. Such abnormalities have been described in many instances with respect to the periodic motor activity of the rectosigmoid area (including the so-called rectal motor complex), that appears either decreased or disorganized^[28-30], the contractile colonic motor response following intravenous cholinergic stimulation, that in results impaired^[31], the early motor response following ingestion of a meal, reported as decreased or absent^[32], the overall daily colonic motility, usually decreased to a lesser or greater extent^[33,34], the daily organization of regular contractile colonic patterns, that is often impaired^[35], and the lack of propulsive response to intraluminal instillation of bisacodyl (a powerful stimulant of mass movements in healthy subjects)^[36].

It is worth noting that neuropathic-type abnormalities in patients with STC are not necessarily confined to the large bowel, but may be also documented in other viscera such as the esophagus^[37,38], the stomach^[39,40], the gallbladder^[41], and especially the small bowel^[42-45], suggesting the presence of a pan-enteric motor disorder in these patients, particularly in those with more severe symptoms.

STC AS AN ENTERIC NEUROPATHY: PATHOLOGICAL EVIDENCE

Most of the pathologic descriptions related to STC are pertinent to the large bowel, and only sporadic reports from other gastrointestinal viscera (terminal ileum) exist. Therefore, the discussion of pathological findings will

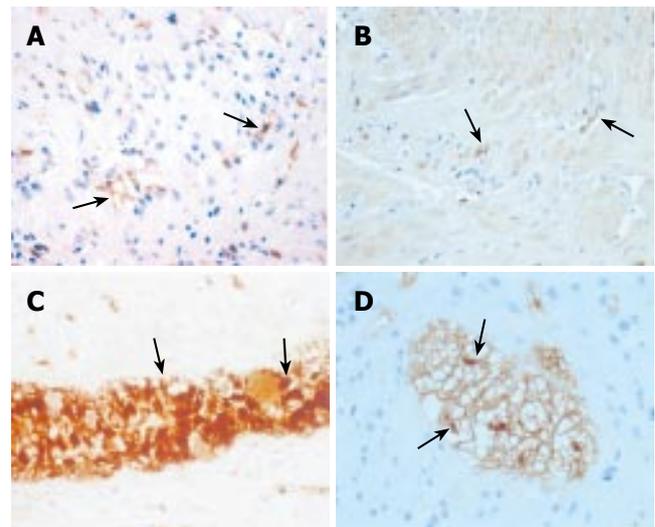


Figure 1 Interstitial cells of Cajal (arrows) at myenteric plexus level in a control subject (A) and in a patient with slow transit constipation (B). Note the considerable decrease of these cells in the patient (CD117, original magnification x 40). Panels C and D show the enteric glial cells (arrows) in the myenteric plexus of a control and of a patients with slow transit constipation, respectively. Note the important reduction of these cells in the patient, where only a few ones may be identified (S100, original magnification x 40).

focus on the colonic studies.

Conventional histology

Most studies employing routine light microscopy have failed to identify consistent abnormalities of the enteric nervous system (ENS) in patients with STC^[46-50], apart from the presence of melanosis coli. However, we have recently demonstrated that melanosis coli *per se* does not have any relationship with colonic ENS abnormalities (in particular with the loss of enteric neurons, as hypothesized in older studies only evaluating the submucosa) in these patients^[51].

Silver staining technique

Morphological abnormalities of colonic innervation have been described in STC patients using the silver staining technique introduced by Smith^[52]. These studies have generally reported a reduction in the total number of argyrophilic neurones and morphological neuronal and/or axonal abnormalities^[53,54]. However, the silver staining technique has been subsequently replaced by more modern and reliable immunohistochemical methods (see below).

Immunohistochemistry

Concerning the hypothesized imbalance of enteric neurotransmitters or the enzyme markers (mostly neuropeptides) in STC, the various studies (using immunostaining, immunoassays, or both methods) have frequently yielded inconsistent results. In fact, looking at the findings related to the most commonly investigated neuropeptides (VIP, substance P, neuropeptide Y and 5-HT), decreased, increased or unchanged levels or immunoreactivity has been described in these patients^[55-59]. Overall, on the basis of the above reports it might be stated that it is unlikely that alterations of the enteric neurotransmitters may play

a major role in the pathophysiology of STC. However, more recent observations suggest that an excessive production of nitric oxide in the colonic myenteric plexus of patients with STC could play a pathophysiological role, concurring in the persistent inhibition of contractions^[60,61].

More consistent results have been reported with respect to enteric neurons, interstitial cells of Cajal (ICC), and enteric glial cells (EGC). In fact, a decrease of enteric neural elements (neurons and/or neurofilaments) seems to be a constant feature in studies evaluating patients with STC severe enough to require surgery for symptoms' relief^[62-66], and these abnormalities are often associated with a reduced number of ICC^[67-71], although this latter finding is not constantly present^[72]. We have recently shown in a relatively large and homogeneous group of patients with severe and intractable STC, compared to age-matched controls, that the ICC are significantly decreased in patients (Figures 1 A and B), that the enteric neuronal loss may be partially due to apoptotic phenomena, and that these patients display a significantly decreased number of EGC with respect to controls (Figures 1 C and D), in both the submucosal and myenteric plexuses^[73].

STC AS AN ENTERIC NEUROPATHY: CLINICAL, INSTRUMENTAL, AND PATHOLOGICAL RELATIONSHIPS

On the basis of the above evidences, it seems now possible to track a link between the clinical picture, represented by severe constipation with heavily delayed colonic transit, often refractory to medical treatment, the instrumental manometric findings, that mostly show impaired motility and propulsive activity of the large bowel (sometimes with the participation of the upper gastrointestinal segments too), and the abnormalities of the colonic ENS.

The neuronal loss is likely to affect the motor activity of the large bowel, reducing the likelihood of enteric neurotransmission. This defect is then strengthened by the concomitant reduction of the number of ICC, a cell population of paramount importance for the correct homeostasis of gastrointestinal motility. In fact, the primary role of ICC as intestinal pacemakers has been established in experimental animal models, where it has been shown that a lack of ICC networks leads to the absence of slow waves and is accompanied by delayed or absent intestinal motility^[74,75]. A decreased ICC function might therefore impair the colonic electrical slow wave activity, thereby affecting the contractile response and causing delayed transit in STC patients. In addition, it has been recently demonstrated that in patients with STC the expression of c-kit mRNA and c-kit protein is significantly decreased compared to controls, suggesting that alterations in the c-kit signal pathway may play an important role in ICC reduction in such patients^[76].

An interesting findings, never described before, was the significant decrease of EGC in both the myenteric and submucous plexuses in STC patients compared to controls. This cell population originates from the neural crest and provides both mechanical and physiological support for neuronal elements^[77]. The chief known function of the

glia in the adult is the formation of myelin sheaths around axons, allowing the fast connections essential for the nervous system function. Moreover, EGC maintain the appropriate concentrations of ions and neurotransmitters in the neuronal environment and are essential regulators of the formation, maintenance and function of synapses, the key functional units of the nervous system^[78,79]. Since EGC are thought to act as intermediaries in enteric neurotransmission^[80], it is likely that their decrease could synergistically act in further weakening the already precarious neuroenteric balance due to the decrease of neuronal elements and ICC found in patients with STC.

STC AS AN ENTERIC NEUROPATHY: CONCLUSIONS

The case for reclassifying STC other than an "idiopathic" or "functional" disorder is built. In fact, as seen above, clinical, instrumental, and pathological evidences exist that all point toward to a (perhaps) more precise definition of this condition as a true enteric neuropathy. It is probably too early to target STC with a different label, but at least we are now aware of some basic pathophysiological mechanisms potentially responsible for the symptoms and the manometric abnormalities found in this condition. Moreover, apart from mere semantic considerations, the demonstration of such background abnormalities might reveal useful for more targeted therapeutic approaches. For instance, in a mouse model the blockage of Kit receptors caused transdifferentiation of intestinal ICC to a smooth muscle phenotype^[81]; if the same would occur in the human colon and if ICC do not die in STC but rather redifferentiate, it might be possible to create conditions that would shift the phenotype back toward ICC.

In conclusion, the advancement of our knowledge of the possible pathophysiological mechanisms of "functional" disorders is important for a more correct clinical and therapeutic approach. Further studies are obviously needed before we can drop the "idiopathic" label from these disorders.

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REVIEW

Intestinal mucosal adaptation

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Abstract

Intestinal failure is a condition characterized by malnutrition and/or dehydration as a result of the inadequate digestion and absorption of nutrients. The most common cause of intestinal failure is short bowel syndrome, which occurs when the functional gut mass is reduced below the level necessary for adequate nutrient and water absorption. This condition may be congenital, or may be acquired as a result of a massive resection of the small bowel. Following resection, the intestine is capable of adaptation in response to enteral nutrients as well as other trophic stimuli. Identifying factors that may enhance the process of intestinal adaptation is an exciting area of research with important potential clinical applications.

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Key words: Small intestine; Transport; Morphology; Resection; Short bowel syndrome; Absorption; Diet; Gene expression; Hyperplasia; Enterocytes

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STRUCTURAL AND FUNCTIONAL ADAPTATION

The intestine has an inherent ability to adapt morphologically and functionally in response to internal and external environmental stimuli. In fact, intestinal adaptation may be considered as a paradigm of gene-environment interactions. The array of phenotypic adaptations includes the modification of brush border membrane (BBM) fluidity and permeability, as well as up- or down-regulation of carrier-mediated transport. In animal models, intestinal adaptation occurs following the loss of a major portion of the small intestine ("short

bowel syndrome", SBS), following chronic ingestion of ethanol, sublethal doses of abdominal irradiation, diabetes, aging, fasting and malnutrition^[1-5]. Following intestinal resection, morphological and functional changes occur depending upon the extent of the intestine removed, the site studied, and the lipid content of the diet^[6]. The increase in nutrient absorption compensates for the loss of absorptive surface area, and minimizes the malabsorption that could otherwise potentially occur. Therefore, intestinal adaptation has important implications in the survival potential and welfare of the host^[7]. However, in some cases such as diabetes, intestinal adaptation may have deleterious effects, with enhanced nutrient uptake exacerbating prevailing hyperglycemia, hyperlipidemia and obesity^[8].

The mechanisms of intestinal adaptation occur at a variety of levels: physiological, cellular and molecular. Signals of adaptation may relate to various hormone levels, transcription factors, ATP levels, or changes in the concentration of luminal solutes^[5]. The signals and mechanisms of the adaptive process may be different for the jejunum and ileum, as well as in the intestinal crypt and villous tip, explaining the site-specific alterations and differences between crypt and villous enterocytes^[3,4].

Rodents are commonly used in well-characterized models of assessing the process of intestinal adaptation^[9]. Following small bowel resection in the rat, the remnant intestinal mucosa undergoes compensatory alterations in an attempt to restore normal absorptive capacity^[10]. Morphologic and functional changes include increases in crypt depth and villous length, enterocyte proliferation, as well as increased electrolyte, glucose and amino acid uptake^[9,10].

The adaptive process has been defined in terms of transport kinetics. Changes usually occur in the value of the maximal transport rate (V_{max}) rather than in the Michaelis affinity (K_m) constant of specific nutrient transporters (sugars and amino acids)^[11,12]. Furthermore, there may be alterations in the passive permeability coefficients of nutrients transported passively such as short-, medium- and long chain fatty acids and cholesterol^[3,4,13]. The increased V_{max} results from either an up-regulation of the total number of transporters, an increased number of transporting mucosal cells, or an increase in the intrinsic activity of the transporter^[14,15]. Intestinal resection also selectively changes the passive permeability properties of the BBM, as demonstrated by the increased uptake of fatty acids following intestinal resection, an increase that was not due to the changes in the mucosal surface area or the effective resistance of

the intestinal unstirred water layer (UWL)^[16]. Indeed, this altered permeability is due to changes in the lipophilic properties of the BBM due to variations in the lipid content of the BBM^[17].

Intestinal adaptation in the rodent model of chronic diabetes involves changes at the transcriptional as well as at the posttranscriptional level, leading to increased Na⁺-coupled sugar absorption^[18]. After inducing acute hyperglycemia in rats, there is rapid up-regulation of glucose transport across the basolateral membrane (BLM) of the enterocytes^[19]. In this model, both the vascular as well as luminal glucose infusion causes an increase in the glucose transport capacity across the BLM^[20]. However, no significant increase in BLM cytochalasin B binding or in GLUT2 protein abundance was observed, suggesting that there may be a post-translational event that increases the number of GLUT2 proteins available for transport, such as the movement of GLUT2 to the BLM from a preformed pool within the enterocyte. Alternatively, the “intrinsic activity” of the transporter may be altered in the absence of changes in the protein abundance. Changes in the intrinsic activity of glucose transporters have been observed with hyperglycemia^[21], diabetes^[22], low luminal glucose concentrations^[14] and following the activation of MAPK and PI3K^[15].

Following extensive intestinal resection, there is hyperplasia of the remaining bowel, which may be accompanied by the enhanced uptake of nutrients^[23]. The alterations in the cell kinetics that result in modification of the nutrition status may be specific or non-specific. *Non-specific mechanisms* involve alterations that result in changes in the intestinal mucosal mass and/or the villous surface area, leading to modifications in the uptake of all nutrients, including those that are absorbed passively^[24]. On the other hand, *specific mechanisms* involve up- or down-regulation of transporters responsible for the uptake of particular nutrients, such as sugars or amino acids^[3,4].

The observation that morphological modifications may accompany intestinal adaptation in the rodent small bowel resection model was first made by Dowling and Booth^[23]. The remaining intestine after resection is hyperplastic, with greater villous height and crypt depth, leading to enhance mucosal surface area. However, while enhanced nutrient absorption is observed, the morphological changes do not necessarily explain the alterations in nutrient uptake. For example, one week after an 80% small bowel resection, the remaining intestine increased its mass to 50%-70% of its pre-resection level, yet the uptake of glucose increased only to approximately 33% of the pre-resection level^[10]. Thus, enhanced nutrient absorption may not be solely explained by intestinal hyperplasia and/or hypertrophy.

It is clear that dynamic morphologic parameters of the intestine may also adapt. For instance, the crypt cell production rates or the enterocyte migration rates change in some situations of intestinal adaptation^[25]. It is important that morphological alterations are considered when estimating the kinetic parameters of absorption. Morphological modifications such as blunting of the mucosal growth or mucosal hyperplasia after intestinal resection are observed when Dexamethasone (Dex) is given subcutaneously^[26]. Both kinetics and dynamic

morphologic parameters are altered in the adaptive process, and the influence of resection on nutrient uptake is due in part to these kinetic alterations. This may be due to the altered cell kinetics changing the population of the enterocytes along the villus, thereby leading to variations in the number of cells with transporter, or the activity of the transporters^[27,28].

Many animal models of intestinal adaptation have been described: glucose uptake has been found to be increased during pregnancy^[24], lactation^[30], with the ingestion of a high carbohydrate diet^[31], hyperglycemia^[32], with diabetes^[33], high alcohol intake^[34] and after intestinal resection^[35]. On the other hand, glucose uptake is decreased with aging^[36], external abdominal radiation^[37] and with the use of total parenteral nutrition^[38]. Most transporters are up-regulated by the levels of dietary substrate levels, whereas toxic substances and essential amino acids have the opposite effect^[9,38-40]. These examples illustrate the diversity and variability of this intestinal adaptive process.

The adaptive response in humans is not well characterized. Increases in nutrient absorption have been documented^[41-43] in humans following resection. The role of morphological changes in this process, however, has not been conclusively demonstrated. Remnant small bowel lengthening and dilatation has been noted in patients with SBS, suggesting morphologic mechanisms in human intestinal adaptation^[44]. However, the mucosal adaptation typical in rodent models is not seen in the human adaptive response^[45,46]. Indeed, several studies have shown that no increases in villous height or crypt depth were detected among patients who underwent intestinal resection, as compared to healthy controls^[42,47].

With the existence of various relevant anatomical, physiological and biochemical differences between the human and rodent gastrointestinal tracts^[48], and a conspicuous lack of comparable human studies, the clinical adequacy of the rat as a model of intestinal adaptation remains to be determined. Although the morphological and functional changes that occur in the rodent following massive small bowel resection have been well characterized^[23,49], direct evidence for similar changes in humans is lacking. Accordingly, caution must be used when attempting to extrapolate findings from rodent studies to the human population. An alternative model, the neonatal piglet, has been used in short bowel studies^[50-52]. The neonatal pig has recently been used to determine the effects of IGF-1 and dietary manipulations in an intestinal resection model^[53,54]. The degree to which the results obtained using this model reflect human findings has yet to be determined, and the rodent remains a popular model for studies of intestinal adaptation.

DIETARY REGULATION

The topic of the dietary regulation of intestinal gene expression has been reviewed^[31,55]. Dietary constituents provide continual environmental signals that elicit the expression of a host of genes that influence intestinal adaptation^[56]. Every day, enterocytes are exposed to different nutrients that vary according to the nutrient intake of the host. For this reason, the intestine must be able to

adapt to variations in the dietary load and composition^[31,57]. The intestine, like many other biological and engineered systems, is quantitatively matched to prevailing peak loads with modest reserve capacities. Indeed, physiological capacities are optimal and most economical if they ascribe to the adage “enough, but not too much”^[57]. Therefore, intestinal enzymes and transporters are characterized by a “safety factor”, a parameter that represents the ratio of its capacity to the load placed on it^[58]. The maintenance of this reserve capacity is biosynthetically costly, but is necessary given the unpredictable nature of dietary contents.

Parenteral vs enteral nutrition

Small bowel atrophy is well characterized in rodent models using total parenteral nutrition^[59-61]. Not surprisingly, the presence of luminal nutrients also contributes greatly to the adaptive process. Intestinal adaptation following massive small bowel resection is limited, but not entirely abolished in the absence of luminal nutrition^[62]. The following sections detail the effects of the type and amount of various luminal nutrients on the adaptive process.

Lipids

Dietary fat content influences the uptake of hexoses and lipids into rabbit jejunum following ileal resection^[16]. More recently, using a rat model of SBS, Sukhotnik *et al* (2003) demonstrated that early feeding of a high fat diet increased lipid absorptive capacity of the intestinal remnant^[63]. The main mechanisms of this effect may be an acceleration of structural intestinal adaptation, resulting in an increased number of enterocytes. However, at the molecular and cellular level, a high fat diet decreased mucosal mRNA levels of the lipid binding protein FAT/CD36 and decreased oleic acid uptake by isolated enterocytes. This is in contrast to what is seen with the liver fatty acid binding protein (L-FABP), a cytosolic lipid binding protein. Mice that were chronically fed a diet enriched in sunflower oil had increased the liver fatty acid binding protein (L-FABP) mRNA levels in their small intestine^[64]. The effect was specific to this gene, as the intestinal fatty acid binding protein (I-FABP) was unaffected.

Not only the amount of fat, but also the type of dietary fat may influence intestinal function. Keelan *et al* (1996) tested the hypothesis that the intestinal morphology and uptake of nutrients after resection of the distal half of the small intestine in rats responds to alterations in the dietary content of saturated (SFA) and polyunsaturated (PUFA) fatty acids^[65]. Adult female Sprague-Dawley rats were subjected to a sham operation or to the surgical resection of the distal half of the small intestine. The animals were fed chow for 3 wk, then either chow or isocaloric semisynthetic SFA or PUFA diets for a further 2 wk. The *in vitro* jejunal uptake of glucose was twice as high in animals that had undergone resection and were fed SFA than in those fed PUFA. It was suggested that SFA was necessary in the diet to ensure that adequate adaptation takes place.

Thiesen and colleagues examined the effect of dietary lipids on lipid uptake in rats post-resection. Intestinal resection had no effect on the mRNA expression of early

response genes (ERGs), proglucagon, or the ileal lipid binding protein (ILBP), but was associated with reduced jejunal mRNA for ornithine decarboxylase (ODC) and for the liver fatty acid binding protein (L-FABP)^[66]. These resection-associated changes in gene expression were not linked with alterations in the intestinal uptake of long chain fatty acids or cholesterol. In animals undergoing intestinal resection and fed SFA or given control vehicle, there was a reduction in jejunal proglucagon mRNA expression as compared to those animals fed chow or PUFA. ODC mRNA expression in the jejunum of resected animals was reduced. Thus, dietary lipids modify the uptake of lipids in resected animals, and ODC and proglucagon may be involved in this adaptive response^[67].

The way by which dietary lipids alter gene expression and consequently change membrane composition and/or nutrient transport may be through the activation of peroxisome proliferator-activated receptors (PPAR), hepatic nuclear factor-4 (HNF-4), nuclear factor κ B (NF κ B), and sterol response element binding proteins 1c (SREBP1c)^[56]. By binding to these transcriptional factors, dietary lipids affect the rate of transcription and consequently the protein synthesis of nutrient transporters^[65,68]. It is also known that PPARs belong to the superfamily of receptors that include the glucocorticosteroid receptor (GR)^[69]. When the locally acting glucocorticosteroid (GC) budesonide was administered concomitantly with SFA diet, the jejunal uptake of glucose was increased but the ileal uptake of fructose was reduced^[70].

It has been suggested that dietary lipids participate in signal transduction involving the activation of second messengers, such as cAMP, Ca²⁺ and diacylglycerol, thereby changing the mRNA expression^[71]. Studies with glycosphingolipid have revealed the importance of these lipids and their metabolites in signaling pathways via the tyrosine kinase-linked receptors, a signal system mediated by protein kinase C (PKC), mitogen activated protein kinase (MAPK), other kinases, as well as mediated by the cytosolic Ca²⁺ concentration^[72]. More recently, additional new signals involved in the adaptive intestinal response 3 days after a 50% intestinal resection have been identified by cDNA microarray analysis, such as small proline-rich protein 2, involved in wound healing; glutathione reductase, a gene involved in intestinal apoptosis; NF-2 family members, also involved in apoptosis; etoposide-induced p53-mediated apoptosis; basic Kruppe-like factor, a transcription factor that activates the promoter for IGF-1; and prothymosin- α , involved in cell proliferation^[73,74]. These observations of altered expression of signals are useful to generate hypotheses that can be tested in future studies to establish whether these signals represent a primary or a secondary event.

The glycosphingolipid, phospholipid, cholesterol and fatty acid composition of plasma membranes may be modified in mammalian cells^[75]. For example, Keelan *et al*. (1990) demonstrated that alterations in dietary fatty acid saturation influence intestinal BBM phospholipid fatty acid composition in rats^[76]. The investigators proposed that the previously reported diet-associated changes in active and passive intestinal transport are due at least in part to these alterations in the fatty acid composition in BBM phospho-

lipids. A diet enriched with SFA is associated with increases in the saturation of BBM phospholipid fatty acids, while a diet enriched with PUFA is associated with an increase in the unsaturation of BBM phospholipid fatty acids^[3,4]. The degree of fatty acid unsaturation or saturation, as well as the cholesterol and ganglioside/glycosphingolipid content, are factors that influence the fluidity of the BBM^[77,78]. Changes in the fluidity of the BBM may alter the permeation of molecules and nutrients through this barrier, as well as the conformation of binding sites on transporter proteins such as SGLT1 and GLUT5^[79]. For example, alterations in BBM fluidity influence the passive uptake of lipids, as well as the carrier-mediated D-glucose uptake^[79,80]. While enhancement of fluidity increases the uptake of lipids, fluidization of BBM from enterocytes located on the villous tip decreases the uptake of D-glucose to levels seen in the BBM from enterocytes located on the crypts^[81]. The explanation for the effect of BBM fluidity on glucose uptake is unknown, but represents a potentially important post-translational process.

The lipid composition of cell membranes alters the passive permeability properties and transporter activity across the membrane^[75]. The altered membrane lipid composition may act in part by changing the viscosity or fluidity of the membrane, including the microenvironment surrounding the transporter. Meddings (1989) compared *in vivo* membrane lipid permeability within the same intestinal region, under conditions where membrane physical properties were radically altered by feeding rats an inhibitor of cholesterol synthesis^[82]. Marked reductions in membrane fluidity were observed due to the replacement of membrane cholesterol with its precursor 7-dehydrocholesterol. Associated with these alterations was a pronounced reduction in membrane lipid permeability. Therefore, BBM membrane lipid permeability, *in vivo*, appears to be correlated with the physical properties of the bilayer.

Recently, two types of specialized microdomains in the BBM have been identified: lipid rafts and caveolae. These regions are important in signal transduction as well as lipid and protein trafficking^[83-85]. They are enriched in saturated fatty acids, cholesterol and gangliosides^[84-86]. Feeding rats a diet enriched with gangliosides increases jejunal glucose uptake^[87]. Feeding a ganglioside-rich diet increases the ganglioside content and decreases the cholesterol content in the intestinal mucosa, plasma, retina and brain^[88]. Similar changes in the lipid composition of intestinal microdomains, or lipid rafts, occur following ganglioside feeding^[89]. Although SGLT1 has been localized to these microdomains in renal epithelial cells^[90], it is not known if sugar transporters reside in intestinal BBM microdomains. If this is the case, local changes in membrane fatty acids may affect the activity of transporter by altering the configuration of the protein, potentially exposing or masking the transporter binding sites and thereby modifying nutrient uptake. In addition, gangliosides may influence intestinal sugar transport *via* their effect on pro-inflammatory mediators, many of which are known to influence intestinal sugar transport^[91-93]. For example, in rats challenged with lipopolysaccharide, ganglioside feeding reduced the production of intestinal platelet activating factor, PGE2, LTB4, as well as reduced plasma levels of IL-1 β and TNF- α ^[94].

Carbohydrates

Dietary carbohydrate may induce the intestinal adaptive response by increasing the abundance of hexose transporters to facilitate a higher rate of sugar absorption^[11]. In a murine model, intestinal glucose uptake was directly correlated with the dietary carbohydrate load^[31,39,95]. The effect of dietary carbohydrate on nutrient transporter abundance has been reported in several animal models. For instance, the abundance of SGLT-1 in BBM and GLUT2 in the BLM were elevated in animals fed a high carbohydrate diet and associated with this enhanced level of protein was an increase in glucose absorption^[19,96,97]. As well, the GLUT5 transporter abundance was elevated with enhanced dietary fructose, leading to increased fructose uptake^[98].

The initiation of the dietary glucose-induced adaptive response occurs in the intestinal crypts, where the transport capacities of the nutrient transporters are programmed^[39,40,95,97]. In this mouse model, phlorizin binding was utilized as a means of measuring the glucose transporter site density. Changing the murine diet from a high to a low carbohydrate regimen reduced the amount of glucose transporter, as estimated from the density of phlorizin binding. The alteration in the density of phlorizin binding was first observed in the crypt cells, and over a three-day period was subsequently seen in the villous tip cells. This suggests that the crypt enterocytes respond to the high carbohydrate diet to increase their phlorizin binding; those cells then migrate up the villous over the next three days, contributing to the process of enhancing glucose uptake.

The enterocytes may adapt to the high carbohydrate diet by increasing the crypt cell turnover rate, enhancing the enterocyte migration rate, as well as by reprogramming the capability of nutrient transporters in the crypts to accommodate to the requirement for higher monosaccharide transporters^[97].

Animals fed a glucose-enriched diet have an increased glucose uptake, resulting from up-regulation of both BBM and BLM glucose transporters^[19,96,99]. The precocious introduction of dietary fructose causes enhanced expression of fructose transporters and fructose transport earlier during development, without changing glucose uptake^[97]. The substrates glucose and fructose are both specific in terms of up-regulation of their corresponding transporters, SGLT1 and GLUT5. Therefore, increasing the sugar composition of the diet results in increases in the transport of these nutrients. In contrast, increases in essential amino acids or other substances that are potentially toxic at high levels (such as iron, calcium or phosphorous) are associated with no change, or even reductions in transport^[38,100].

Furthermore, in many cases other nutrients may be equal or even more potent inducers of the transporter than its specific substrate. For example, young animals fed a diet enriched with polyunsaturated fatty acids (PUFA) have a decline in glucose uptake, as compared to animals fed a saturated fatty acid (SFA) enriched diet^[70,101,102]. Similarly, Vine *et al* (2002) studied the effect of various fatty acids on the passive and active transport properties of rat jejunum, and found that an SFA-enriched diet increased Na⁺-dependent glucose uptake when compared to a diet

enriched with n6 PUFA^[103]. In contrast, in aged rats, glucose uptake is increased by PUFA and not by SFA^[36].

Dietary fiber also modulates intestinal nutrient uptake. For example, a diet enriched with fermentable fiber increased glucose uptake and GLUT2 transporter abundance in dogs^[104]. *In vitro* studies, in which rat intestinal tissue was incubated with β -glucan isolated from barley or oats, show reductions in the uptake of stearic and linoleic acids (Drozowski *et al*, 2005, unpublished observations). Furthermore, many studies have investigated the effect of TPN supplemented with short chain fatty acids, the products of fiber fermentation. Increases in glucose uptake, GLUT2 mRNA and protein, and intestinal morphology were seen in normal rats as well as in rats following intestinal resection^[105-108].

Protein

Dietary protein also has an impact on the intestinal morphology and active amino acid transport^[40,109,110]. Both *in vitro*^[110] and *in vivo*^[109] rat experiments have shown that a high protein diet increases amino acid uptake in the jejunum. An alteration in the amount of dietary protein induces reversible adaptation of the non-essential amino acid transport rate^[111]. Feeding a high protein diet to mice induces a 77%-81% increment in the uptake of non-essential amino acids^[40], yet only a 32%-61% increase for essential amino acids. On the other hand, a protein-deficient regimen reduces uptake of non-essential amino acids, such as aspartate and proline, and maintains or increases uptake for essential amino acids and alanine. Thus, the nature of the adaptive response depends upon the type of amino acid and the needs of the animal.

Glutamine is a key metabolic fuel for enterocytes, mediating cellular nucleic acid synthesis and proliferation. Parenterally fed rats demonstrate decreased atrophy of the intestinal mucosa following glutamine supplementation^[112]. Glutamine administration also normalizes the reduced levels of intestinal adaptation in rats receiving total parenteral nutrition (TPN) following intestinal resection^[113]. It is noteworthy that some studies of oral glutamine supplementation in the rat have failed to document more than temporary mucosal proliferation^[114]. This indicates that mechanistic differences that are intrinsic to the method of glutamine administration may exist, and suggests that these may be significant in regulating the adaptive response.

Other amino acids may inhibit intestinal adaptation. Sukhotnik *et al* (2005) examined the effects of the parenteral administration of the nitric oxide precursor arginine to rats following 75% small bowel resection^[115]. Arginine supplementation was associated with lower cell proliferation indexes and greater enterocyte apoptosis. This observation led the investigators to conclude that arginine inhibits structural intestinal adaptation.

Polyamines

Polyamines are found in all eukaryotic cells^[116], and they play an important role in growth and differentiation^[117]. Polyamines are obtained either from the diet, or via synthesis from ornithine^[118]. Uda *et al* (2002) demonstrated that luminal perfusions of polyamines rapidly (in less

than 5 min) enhance intestinal glucose uptake in rats, and increase BBM SGLT1 protein^[119].

Polyamine synthesis or uptake may be an important event that initiates the adaptive hyperplasia seen in the intestinal remnant after partial small bowel resection. Enteral diets supplemented with ornithine alpha-ketoglutarate (OKG), a precursor for arginine, glutamine and polyamines, enhances intestinal adaptation in models of intestinal resection^[120,121]. Indeed, studies by both Tappenden *et al*^[122] and Thiesen *et al*^[66] suggest that ornithine decarboxylase (ODC), a key enzyme in polyamine synthesis, may mediate the adaptive process in rats that is stimulated by the administration of either glucocorticosteroids or short chain fatty acids to rats following intestinal resection.

The role of polyamines in the adaptation of the intestine during development has also been studied. Wild *et al* (2005, unpublished observations) showed that in postnatal rats, oral spermidine treatment resulted in the precocious expression of the intestinal sugar transporters (SGLT1, GLUT2 and GLUT5), as well as ODC. This led the investigators to conclude that oral polyamines induce precocious maturation of sugar transporters, which may be mediated by alterations in ODC gene expression.

HORMONAL REGULATION

Glucocorticosteroids

In a model of extensive intestinal resection (50% enterectomy), the proximal and distal intestinal remnants were adequate to assess the morphology and function at these sites^[11,112]. The glucocorticosteroid prednisone had no effect on the intestinal uptake of glucose or fructose in these resected animals^[70]. In contrast, the locally acting steroid budesonide increased by over 120% the value of the jejunal Vmax for the uptake of glucose, and increased by over 150% the ileal uptake of fructose. The protein abundance and mRNA expression of SGLT1, GLUT5, GLUT2 and Na⁺/K⁺ APTase α 1 and β 1 cannot explain the enhancing effect of budesonide on glucose and fructose uptake. Budesonide, prednisone and dexamethasone reduced the jejunal expression of the early response gene c-jun. In resected animals, the abundance of the mRNA of ODC in the jejunum was reduced, and glucocorticosteroids (GC) reduced the jejunal expression of the mRNA of proglucagon. These data suggest that the enhancing influence of GC on sugar uptake in resected animals may be achieved by post-translational processes involving signalling with c-jun, ODC and proglucagon, or other as yet unknown signals.

In contrast, the uptake of D-fructose by GLUT5 was similarly increased with budesonide and with prednisone. The increases in the uptake of fructose were not due to variations in the weight of the intestinal mucosa, food intake, or in GLUT5 protein or mRNA expression. There were no steroid-associated changes in mRNA expression of c-myc, c-jun, c-fos, of proglucagon, or of selected cytokines. However, the abundance of ileal ODC mRNA was increased with prednisone. Giving budesonide or prednisone to post-weaning rats for four weeks in doses equivalent to those used in clinical practice increases

fructose but not glucose uptake. This enhanced uptake of fructose was likely regulated by post-translational processes^[70].

Growth hormone

Growth hormone (GH) has been suggested as possessing pro-adaptive properties^[123]. In rats and piglets, GH administration results in an increase in small bowel length and function per unit length^[124]. Hypophysectomized rats undergo mucosal hypoplasia of the small bowel, as well as a reduced adaptive response following resection that is restored by GH^[125]. In contrast, transgenic mice expressing elevated levels of GH show hypertrophy of the small intestine^[124]. IGF-1 expression in the small bowel is regulated by GH and is believed to induce enterotrophic effects following resection^[113,126]. In a rat model of SBS, acute IGF-1 treatment of TPN fed rats produced sustained jejunal hyperplasia, and facilitated weaning from parenteral to enteral nutrition^[127]. GH administration to normal rats has been reported to have positive effects on mucosal growth and intestinal adaptation following massive resection^[128], although contradictory data exists^[129,130]. Human and rabbit studies have indicated that increased nutrient transport activity devoid of morphologic changes may be the method of GH-induced intestinal adaptation^[131].

GH administration has been shown to inhibit the liberation of glutamine from muscle during catabolic states in humans^[132], suggesting a possible role for combined GH and glutamine provision in adaptive bowel enhancement. Trials investigating any such synergism in the rat have yielded conflicting results. Some studies have failed to demonstrate an additive effect of GH and glutamine in the enhancement of post-resection intestinal adaptation^[133], while others have documented a positive synergistic effect^[134].

The mechanisms of action by which GH and/or glutamine may enhance the human adaptive process cannot be clearly surmised from the existing rodent data. Many studies have inherent deficits in terms of nutrient controls that could have contributed to the conflicting outcomes which have been described. The trophic effects of enteral nutrition on the adaptation process are well known^[3]. Studies evaluating the contribution of non-specific, nutrient-derived augmentation of the adaptation process, as well as the mechanisms of any such nutrient factor interactions, may be useful in defining more accurate and therapeutically applicable results.

Animal studies have confirmed the enhancing effect of GH on nutrient absorption^[123,124]. For example, GH has been shown to enhance the absorption of amino acids using *ex vivo* human BBM vesicles^[134]. An intestinal mucosal GH receptor has been described in rats and humans^[135], and GH promotes cell differentiation and clonal expansion of these differentiated cells^[136].

The use of growth hormone in human clinical studies has been investigated. It has been suggested that the efficacy of GH and/or glutamine therapy in the adaptive response of the small bowel may be based mainly upon the clinical status of the patient (for example, the

presence of a portion of the colon in continuity with the remaining resected small intestine)^[137]. Evaluation of the effect of such variables in the rat may facilitate further understanding of the pathology and physiology of the bowel adaptation process, as well as more clearly defining positive predictive indicators of the bowel's ability to be rehabilitated. Furthermore, existing human data has indicated that the administration of high concentrations of GH can actually increase patient morbidity and mortality^[138], demonstrating a primary need for equivalent clinical research in the testing of these factors.

In home parenteral nutrition (HPN)-dependent patients with SBS, the use of high dose recombinant human GH (0.4 mg/kg per day) in controlled^[138,139] and uncontrolled studies^[140] has led to variable results. These patients were given glutamine supplements by mouth or parenterally, and their diet was modified. In the randomized and placebo-controlled study of Scolapio *et al.*^[139], the subjects ingested a standardized 1500 kcal/d diet, which is clearly different from the hyperphagic diet consumed by many SBS patients^[140], and which may contribute to the physiological adaptation that occurs in the remaining intestine after extensive resection. It is unclear whether glutamine is beneficial for the adaptive response in humans, and in rat models of SBS, it is unclear whether glutamine supplementation is efficacious for the adaptive process^[2,141]. Furthermore, both a hyperphagic diet and the absence of malnutrition are needed for humans to achieve optimal intestinal adaptation^[44,142].

When HPN-dependent patients with SBS were provided a usual *ad libitum* hyperphagic diet, and given low doses of GH (0.05 mg/kg per day) for three weeks, there was significant improvement in the intestinal absorption of energy (15% ± 5%), nitrogen (14% ± 6%) and carbohydrate (10% ± 4%)^[143]. The increased food absorption represented 37% ± 16% of total parenteral energy delivery. Body weight, lean body mass, D-xylose absorption, insulin-like growth factor 1, and insulin-like growth factor binding protein 3 increased, whereas uptake of GH binding protein decreased. During treatment with GH, improvement in net intestinal absorption compared with placebo was 427 ± 87 kcal/d, representing 19% ± 8% of the total energy expenditure required to obtain energy balance equilibrium in patients with SBS^[140].

A review of the literature in this area by Matarese *et al.*^[144] noted that there were differences in gastrointestinal (GI) anatomy, dietary compliance, nutritional status, presence of mucosal disease, and diagnosis both within and between the studies. They concluded that “*administering recombinant human growth hormone alone or together with glutamine with or without a modified diet may be of benefit when the appropriate patients are selected for treatment*”.

Insulin-like growth factor 1

Insulin-like growth factor 1 (IGF-1) also proved to be efficient in increasing intestinal adaptation following resection in rats. IGF-1 treatment following 70% jejuno-ileal resection attenuated fat and amino acid malabsorption^[145] and increased total gut weight by up to 21%. The IGF-1 receptor was increased in the jejunum and

colon due to resection. Resection also increased circulating IGF-binding proteins (IGFBP). IGF-1 treatment had no effect on IGF-1 mRNA or IGF-1 receptor density, but increased IGFBP5 in the jejunum. This increase in IGFBP5 was correlated with jejunal growth after IGF-1 treatment^[146].

More recently, a study was conducted to determine the effect of IGF-1 on enterocyte kinetics following intestinal resection^[147]. IGF-I treatment in resected rats significantly increased jejunal mucosal mass by 20% and mucosal concentrations of protein and DNA by 36% and 33%, respectively, above the response to resection alone. These changes reflected an increase in enterocyte proliferation and an expansion of the proliferative compartment in the crypt. No further decrease in enterocyte apoptosis and increase in enterocyte migration were observed.

IGF-I treatment may also facilitate weaning from parenteral to enteral nutrition. After a 60% jejunioileal resection plus cecectomy, rats treated with recombinant human IGF-I (3 mg/kg body weight) or control vehicle were maintained exclusively with TPN for 4 d and were then transitioned to oral feeding. TPN and IGF-I were stopped 7 d after resection and rats were maintained with oral feeding for 10 more days. Acute IGF-I treatment induced sustained jejunal hyperplasia, as demonstrated from the presence of greater concentrations of both jejunal mucosal protein and DNA, and was associated with the maintenance of a greater body weight and serum IGF-I concentrations^[127].

A study was done using male transgenic mice with targeted smooth muscle IGF-1 overexpression^[148]. These animals and non-transgenic littermates underwent 50% proximal small bowel resection. The results showed that growth factor over-expression led to a unique mucosal response characterized by a persistent increase in remnant intestinal length and an increase in mucosal surface area. Therefore, IGF-1 signaling from within the muscle layer may be important in resection-induced intestinal adaptation.

Epidermal growth factor

Epidermal growth factor (EGF) up-regulates intestinal nutrient transport^[149]. This effect is mediated by PKC and PI3K^[150] and involves the redistribution of SGLT1 from microsomal pools to the BBM^[151]. After massive intestinal resection, endogenous EGF is increased in the saliva and is decreased in the urine^[152]. EGF stimulates intestinal adaptation after intestinal resection: the BBM surface area and the total absorptive area increased until d 10, and EGF treatment induced a further increase in BBM surface area^[153]. In a study by O'Brien and colleagues^[154], mice underwent a 50% small bowel resection or sham operation, and were then given orally an epidermal growth factor receptor (EGFR) inhibitor (ZD1839, 50 mg/kg per day) or control vehicle for 3 d. ZD1839 prevented EGFR activation, as well as the normal postresection increases in ileal wet weight, villus height, and crypt depth. Enterocyte proliferation was reduced two-fold in the resection group by ZD1839. These results more directly confirm the requirement of a functional EGFR as a mediator of the

postresection adaptation response. Interestingly, previous work has demonstrated that the EGFR is predominantly located on the BLM of enterocytes^[155], but after small bowel resection the EGFR shows redistribution from the BLM to the BBM, with no change in the total amount of EGFR^[156]. It is not known how this redistribution occurs. This is an important point, since modification of this process may represent a useful means to accelerate the intestinal adaptive process.

In a study by Knott *et al*^[157], laser capture microdissection (LCM) microscopy was used to elucidate the specific cellular compartment(s) responsible for postresection changes in EGFR expression. Mice underwent a 50% proximal resection or sham operation, and after three days frozen sections were taken from the remnant ileum. Individual cells from the villi, crypt, muscularis and mesenchymal compartments were isolated. EGFR mRNA expression for each cell compartment was quantified using real-time reverse transcription polymerase chain reaction (RT-PCR). EGFR expression was increased two-fold in the crypt after resection, directly correlating with the zone of cell proliferation. This supports the hypothesis that EGFR signaling is crucial for the mitogenic stimulus for adaptation. The additional finding of increased EGFR expression in the muscular compartment is novel, and may imply a role for EGFR in the muscular hyperplasia seen after massive small bowel resection. As noted previously, it is of interest that the muscle layer also appears to play a role in the adaptive response to IGF-1^[148].

The treatment of resected rats with EGF has been studied. In a study by Sham *et al*^[158], male juvenile rats underwent either transection or ileocecal resection leaving a 20-cm jejunal remnant. Resected animals were treated orally with placebo or recombinant human EGF. Resected EGF-treated animals lost significantly less weight than those in the transection group, absorbed significantly more 3-O-methylglucose, and had reduced intestinal permeability as determined by the lactulose/mannitol ratio. Work by Chung *et al*^[159] using rabbits showed that intestinal resection altered SGLT1 mRNA and protein expression along the crypt-villous axis, with expression being highest in the mid-villous region. Oral EGF normalized SGLT1 expression, resulting in a gradient of increasing expression from the base of the villus to the villous tip.

More recently, Nakai and colleagues^[160] investigated the role of EGF in stimulating intestinal adaptation following small bowel transplantation. Treatment of rats with EGF (intraperitoneally for three days) following intestinal transplantation resulted in increased glucose absorption, SGLT1 abundance and the villous height and crypt depth in the graft. This has not yet been studied in humans.

Keratinocyte growth factor

In a study by Yang *et al*^[161], adult C57BL/6J mice were randomized to a 55% mid-small bowel resection, resection with keratinocyte growth factor (KGF) administration (SBSKGF), or a sham-operated (control) group, and were killed at d 7. Ussing chamber studies showed that KGF increased the net transepithelial absorption of 3-O-methyl glucose as well as sodium-coupled alanine absorption, but

had no effect on epithelial permeability barrier function. Epithelial cells were separated along the crypt-villous axis with LCM, and epithelial KGF receptor (KGFR) mRNA abundance was studied using real time RT-PCR. KGF up-regulated KGFR mRNA abundance, predominately in the crypt and the lower portion of the villus.

Leptin

Leptin plays an important role in the regulation of body fat and satiety (reviewed in^[162]). Leptin reduces food intake^[163] and leptin-deficient mice develop obesity^[164]. Leptin may be a potential growth factor for the normal rat small intestine. The effect of 14 d of parenteral leptin administration (2 µg/kg per day) to rats following 80% small bowel resection was studied. Leptin was associated with a 44% increase in galactose absorption and a 14% increase in GLUT-5 abundance, but with no change in DNA content or in SGLT abundance. These findings suggest that leptin may potentially be clinically useful in patients with impaired intestinal function^[165].

Ghrelin

Ghrelin is a gastric hormone that is released in response to enteral nutrients. It has an opposite effect when compared to leptin, as it stimulates food intake^[166]. The role of ghrelin in intestinal adaptation is unknown.

Glucagon-like peptide 2

Animal studies have demonstrated a potential role for GLP-2 in the adaptive response following intestinal resection^[147]. Several investigators have demonstrated increases in plasma GLP-2 levels following intestinal resection in rats^[167-169]. In a study by Dahly *et al.*^[147], rats were subjected to 70% midjejunioileal resection or ileal transection, and were maintained with TPN or oral feeding. Resection-induced adaptive growth in TPN- and orally-fed rats was associated with a significant positive correlation between increases in plasma bioactive GLP-2 and proglucagon mRNA abundance in the colon of TPN-fed rats and in the ileum of orally fed rats. While these increases were transient in the TPN-fed group, luminal nutrients produced a sustained increase detected at 3 and 7 d post-resection. These data support a significant role for endogenous GLP-2 in the adaptive response to mid-small bowel resection in both TPN and orally fed rats^[147].

Recently, further correlations between post-resection GLP-2 levels, morphological indices, crypt cell proliferation rates and nutrient absorption have been made^[170]. In this study, an inverse correlation was found between post-prandial GLP-2 levels and fat or protein absorption as assessed by a 48 h balance study. These results, along with data obtained from studies showing that GLP-2 immunoneutralization inhibits post-resection adaptation^[171], further implicate GLP-2 as a post-resection mediator of adaptation.

GLP-2 administration in rats increases the adaptive response to massive intestinal resection^[172]. In this study, Sprague-Dawley rats were divided into two groups, with a 75% mid-jejunum-ileum resection and a sham operated groups. Animals were treated with 0.1 µg/g GLP-2 analog (protease resistant human GLP-2) or placebo given

subcutaneously twice daily for 21 d. The total weight of the rats and the mucosal mass per centimeter were compared between the groups. Administration of this peptide or its analogs was associated with an increase of the mucosal mass in the proximal jejunum and terminal ileum. The absorption and urinary excretion of oral D-xylose is proportional to intestinal mucosal surface area and transit time. While resection reduced D-xylose excretion and GLP-2 restored D-xylose excretion to levels above control values within 21 d of administration. This indicates that GLP-2 has a positive effect on intestinal morphology and absorptive function following resection.

More recently, Martin *et al.*^[173] investigated the effects of GLP-2 in a TPN-supported model of experimental short bowel syndrome. Juvenile Sprague-Dawley rats underwent a 90% small intestinal resection and were randomized to three groups: enteral diet and intravenous saline infusion, TPN only, and TPN plus 10 µg/kg per hour^{GLP-2}. TPN plus GLP-2 treatment resulted in increased bowel and body weight, villus height, intestinal mucosal surface area, and crypt cell proliferation. Intestinal permeability tests showed that GLP-2 reduced the lactulose-mannitol ratio indicating that GLP-2 lowered intestinal permeability when compared with the TPN alone. GLP-2 increased serum GLP-2 levels and intestinal SGLT-1 protein abundance compared with either TPN or enteral groups. This study demonstrates that GLP-2 is capable of stimulating intestinal adaptation in the absence of enteral feeding,

Because a number of hormones and growth factors have been shown to influence intestinal function, Washizawa *et al.*^[174] compared the effects of GLP-2, GH and KGF on markers of gut adaptation following massive small bowel resection (MSBR). KGF increased goblet cell numbers and TTF3, a cytoprotective trefoil peptide, in the small bowel and the colon. They also observed that while both GH and KGF increased colonic mucosal growth, GLP-2 exerted superior trophic effects on jejunal growth. GLP-2 also increased the glutathione/glutathione disulfide ratio, resulting in improved mucosal glutathione redox status throughout the bowel. Because of the differential effects of GLP-2, GH and KGF on gut adaptation following MSBR, the authors conclude that a combination of these agents may be most beneficial.

A pilot study to determine the efficacy of GLP-2 in patients with SBS has been completed. A non-placebo controlled study was conducted in 8 patients with SBS with an end-enterostomy type of anastomosis (6 had Crohn's disease and 4 were not receiving HPN)^[175]. Treatment with GLP-2 (400 µg subcutaneously twice a day for 35 d) increased intestinal absorption of energy, body weight, and lean body mass. Crypt depth and villous height were also increased in 5 and 6 patients, respectively.

A review by Jeppesen^[176] on the role of GLP-2 in the treatment of SBS concludes that "Currently, hormonal therapy in short-bowel patients should be considered experimental and it is only recommended in research studies. The optimal duration and concentration requirements for GLP-2 to induce beneficial effects on intestinal secretion, motility, morphology and most importantly absorption, are not known. Optimal dosage and administration of this new treatment to short-bowel patients may eventually result in long-term improvements in nutritional status and independence of

parenteral nutrition in a larger fraction of short-bowel patients”.

SIGNALS OF INTESTINAL ADAPTATION

A number of studies have investigated the signals involved in intestinal adaptation using animal models of intestinal resection. Dodson *et al*^[177] identified three subsets of genes that were up-regulated by constructing a cDNA library from the remnant ileum of resected rats. This library was screened, and subtractive hybridization was used to identify genes that were induced following resection. These included genes involved with regulating the absorption and metabolism of nutrients. For example, L-FABP, apolipoprotein A-IV, cellular retinal binding protein II and ileal lipid binding protein were identified as genes that were induced following 70% intestinal resection in rats. Genes involved in cell cycle regulation were also identified. For example, phosphorylation and dephosphorylation are important regulators of the cell cycle, and PP1 δ , a subunit of a serine/threonine phosphatase was indeed up-regulated. Grp78, a member of the heat shock protein family was also increased. Grp78 resides in the ER and acts as a chaperone during protein assembly and transport. It may also have a protective role, and prevent apoptosis as a way of promoting the proliferative response following intestinal resection^[178,179].

Rubin *et al*^[180] further characterized the molecular and cellular mechanisms following 70% resection in rats. An immediate early gene, PC4/TIS7, was markedly increased soon after resection, with levels returning back to normal by one week post-resection. Although the function of this protein is unknown, it may be related to cytodifferentiation as it is expressed only in the villus and not in the crypts.

Erwin *et al*^[74] used cDNA microarrays to gain insight into the mechanism of intestinal adaptation. Mice underwent a 50% intestinal resection and three days afterwards RNA was extracted from the remnant ileum. Multiple genes were induced, and they divided these into four categories: (1) Apoptosis, DNA synthesis, repair and recombination (10 genes); (2) Oncogenes, tumor suppressors, cell cycle regulators (3 genes); (3) Stress response, ion channels and transport (4 genes); (4) Transcription factors and general DNA-binding proteins (1 gene). Many of the genes (ODC, c-neu, glucose-related protein, IGFBP-4) that were identified agreed with the results of other studies of intestinal resection. For example, ODC was increased in this study, and this agrees with previous findings that showed ODC to be involved in the adaptive process^[66,122,181]. Some new factors were also identified including glutathione reductase (involved in apoptosis), Basic Kruppel-like factor (transcriptional regulator that activates the IGF promoter), prothymosin- α (associated with increased cell proliferation), and eteptide induced p53 responsive mRNA (stress response protein involved in p53 mediated apoptosis).

Stern *et al*^[73] performed a similar analysis of gene expression following 50% intestinal resection in rats. The gene with the largest increase was identified as *spr2*, a novel gene unknown previously to be involved in intestinal adaptation. EGF administration post-resection further increased *spr2* expression and enhanced the

adaptive response. This protein plays a role in the terminal differentiation of stratified squamous epithelium. Its role in the intestinal epithelium is unclear and warrants further investigation.

Finally, a variety of other signals have been described as possibly playing a role in the process of intestinal adaptation. These include prostanoids^[182], uncoupling proteins^[183], peroxisome proliferation-activated receptor α (PPAR α)^[184], transforming growth factor- α ^[185], SPARC (secreted protein, acidic and rich in cysteine^[186], Bcl-2^[187], endothelin-1^[188], erythropoietin^[189], the GATA family of zinc finger transcription factors^[190], hepatocyte growth factor^[191], the early response genes (ERG)^[192], PC4/TIS7^[180] and epimorphin^[193]. More recently, augmented Wnt signaling has been shown to enhance the adaptive response to massive small bowel resection^[194]. Several of these signals may be useful to modify in a clinical setting to enhance the intestinal adaptive response.

Microarray technology is a powerful tool that is constantly developing into a more sophisticated technique of identifying novel genes involved in physiological processes. Intestinal adaptation awaits further characterization by hypothesis-testing studies. From the information that is available at this time, it is clear that genes regulating the cell cycle, proliferation, differentiation and apoptosis are important components of the adaptive process.

CONCLUSION

The process of intestinal adaptation is complex and multifaceted. Although a number of trophic nutrients and non-nutritive factors have been identified in animal studies, successful and reproducible clinical trials are lacking. However, the functional adaptation of segmental bowel grafts has been studied. Benedetti *et al* (2001) reported a case study in which a segmental graft from a living donor demonstrated pregressive functional adaptation, as assessed by water and D-xylose absorption as well as fecal fat studies^[195]. Morphological adaptation has been documented in three patients following successful intestinal transplantation^[196]. In fact, more than 50% increases in villous height and absorptive surface area were seen at 6 mo, suggesting that functional changes in intestinal function are associated with morphological changes. Therefore, while human data is limited there is evidence indicating that the adaptive process occurs. Still, additional human studies are required before the clinical implications of the animal data can truly be established. Understanding the mechanisms underlying the adaptive process in humans may direct research toward strategies that maximize intestinal function and impart a true clinical benefit to patients with short bowel syndrome.

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REVIEW

Novel strategies for the treatment of inflammatory bowel disease: Selective inhibition of cytokines and adhesion molecules

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Abstract

The etiology of inflammatory bowel disease (IBD) has not yet been clarified and immunosuppressive agents which non-specifically reduce inflammation and immunity have been used in the conventional therapies for IBD. Evidence indicates that a dysregulation of mucosal immunity in the gut of IBD causes an overproduction of inflammatory cytokines and trafficking of effector leukocytes into the bowel, thus leading to an uncontrolled intestinal inflammation. Such recent advances in the understanding of the pathogenesis of IBD created a recent trend of novel biological therapies which specifically inhibit the molecules involved in the inflammatory cascade. Major targets for such treatment are inflammatory cytokines and their receptors, and adhesion molecules. A chimeric anti-TNF- α monoclonal antibody, infliximab, has become a standard therapy for CD and it is also likely to be beneficial for UC. Several anti-TNF reagents have been developed but most of them seem to not be as efficacious as infliximab. A humanized anti-TNF monoclonal antibody, adalimumab may be useful for the treatment of patients who lost responsiveness or developed intolerance to infliximab. Antibodies against IL-12 p40 and IL-6 receptor could be alternative new anti-cytokine therapies for IBD. Anti-interferon- γ and anti-CD25 therapies were developed, but the benefit of these agents has not yet been established. The selective blocking of migration of leukocytes into intestine seems to be a nice approach. Antibodies against $\alpha 4$ integrin and $\alpha 4\beta 7$ integrin showed benefit for IBD. Antisense oligonucleotide of intercellular adhesion molecule 1 (ICAM-1) may be efficacious for IBD. Clinical trials of such compounds have been either recently reported or are currently underway. In this article, we review the efficacy and safety of such novel biological therapies for IBD.

INTRODUCTION

Although the etiology of inflammatory bowel disease (IBD), such as, Crohn's disease (CD) and ulcerative colitis (UC), has not yet been fully addressed, there has been remarkable progress in the understanding of this field in the past decade. In normal bowels, the immune reaction is sophisticatedly regulated while keeping a balance between the effectors and the regulators, and as a result, the homeostasis of the gut is maintained. A lot of evidences indicate that mucosal immunity is dysregulated in the bowel of IBD^[1]. Two forms of IBD show distinct profiles of T cell mediated immunity. In the gut of CD, a strong Th1 reaction is induced, while the Th2 response is upregulated in the colon of UC^[2]. Particularly in CD, it is evident that Th1 dominant immunity plays an important role in the pathogenesis. In UC, although the relevance of elevated Th2 cytokines to the colonic inflammation has not yet been clarified, an increased proinflammatory cytokine production is also observed which seems to be related to the inflammation. For the development of intestinal inflammation, leukocyte trafficking to the gut is an important step^[3]. Adhesion molecules, such as integrins, mediate the selective binding between the leukocytes and the endothelial cells and thus the migration of leukocytes into the normal and inflamed intestine. The main classical medical treatments for IBD are steroids and immunosuppressive agents which non-specifically reduce immunity and inflammation. Recent advances in the understanding of the mechanism of bowel inflammation have led to a recent trend in the development of biological therapies which selectively inhibit the action of molecules essential to the inflammatory process. Major targets for such therapies are inflammatory cytokines and their receptors, and adhesion molecules. Re-

cently, numerous challenges have been performed to generate anti-cytokine and anti-integrin compounds to treat IBD. This article reviews the efficacy and safety of such novel biological therapies for IBD targeting cytokines, cytokine receptors and adhesion molecules.

INHIBITION OF TNF

TNF- α is a proinflammatory cytokine which is abundantly expressed in the gut of CD^[4,6]. In animal models of experimental colitis, treatment with anti-TNF- α antibody has been shown to be effective in the suppression of intestinal inflammation^[7,8]. As a result, this cytokine was considered to be an attractive target for the treatment of IBD and several anti-TNF reagents have thus been developed. These reagents include infliximab, CDP571, CDP870, etanercept, oncept and adalimumab. Infliximab is a chimeric IgG1 monoclonal antibody against TNF- α , which was created in late 1980s, and it has been demonstrated to be effective in reducing intestinal inflammation in CD as described below. Most of the other anti-TNF reagents are modified by a reduction of the mouse peptide sequence or are completely humanized in order to reduce the immunogenicity. Not all of the other anti-TNF reagents have been proven to be as effective in the treatment of CD as infliximab, and the efficacy of such reagents seems to be dependent, not only on the ability to neutralize soluble TNF- α , but also on the capacity to bind to the membrane-bound TNF- α on the cell surface, thereby mediating the apoptosis of the effector cells^[9].

Infliximab

Infliximab was demonstrated to be effective in both the induction and maintenance therapy for refractory luminal and fistulizing CD. In a randomized double-blind placebo-controlled trial, 108 patients with moderate-to-severe CD which is resistant to conventional therapy, were treated with the single intravenous infusion of either placebo or infliximab at a dose of 5 mg/kg, 10 mg/kg or 20 mg/kg. The rates of the clinical response at 4 wk were 81% for infliximab 5 mg/kg, 50% for infliximab 10 mg/kg and 64% for infliximab 20 mg/kg, all of which were significantly higher than that for the placebo-treated group. The clinical remission rate at 4 wk was also significantly higher in the infliximab-treated group than that in the placebo-treated group (33% *vs* 4%)^[10]. In a randomized, double-blind, placebo-controlled trial for the treatment of fistulizing disease, 94 CD patients with draining abdominal and perianal fistulas refractory to conventional therapy were treated with three intravenous infusions at wk 0, 2 and 6 of either a placebo or infliximab at a dose of 5 mg/kg or 10 mg/kg. The response rates were significantly greater in the infliximab 5 mg/kg group (68%) and in the infliximab 10 mg/kg group (56%) than that in the placebo-treated group (26%). The rates of a complete closure of the fistulas were also significantly higher in the infliximab 5 mg/kg group (55%) and in the infliximab 10 mg/kg group (38%) than in the placebo-treated group (13%)^[11]. The effectiveness of infliximab for the maintenance therapy for inflammatory CD was assessed in a large trial called ACCENT I. Three hundred and thirty-five responders to a single infusion of infliximab

were subsequently treated with 5 mg/kg infliximab at wk 2 and 6, followed by infusions of either 5 mg/kg or 10 mg/kg infliximab once every 8 wk until wk 54, or they were treated with placebo at wk 2 and 6, and subsequently every 8 wk. The rates of clinical response and remission at wk 30 and 54 was significantly greater in both groups receiving 5 mg/kg and 10 mg/kg infliximab every 8 wk than those in the placebo-treated group^[12]. In addition, an analysis comparing the scheduled and episodic treatment strategies of infliximab for CD was conducted based on the ACCENT I data. The efficacy of the scheduled therapy was better than episodic strategy in terms of CDAI score, clinical remission and response rates, improvement in IBDQ score, mucosal healing and CD-related hospitalization and surgery^[13]. For an evaluation of the infliximab maintenance therapy for fistulizing CD, ACCENT II trial was conducted. One hundred and ninety-six CD patients with draining perianal and enterocutaneous fistulas who responded to the induction therapy with three infusions of 5 mg/kg infliximab at wk 0, 2 and 6 received either a placebo or 5 mg/kg infliximab every 8 wk. The median time to the loss of response, response rate and complete fistula closure rate at wk 54 in the infliximab maintenance group were significantly greater than those in the placebo group^[14].

Regarding the safety of infliximab treatment, it is well tolerated in the majority of the patients. In randomized controlled clinical trials, the rates of adverse events occurring in infliximab-treated patients were comparable to those in placebo-treated patients^[10-12,14]. Serious side effects, however, have been reported and attention must be paid to the possible occurrence of serious infections and autoimmune disorders, as well as the theoretical threat of cancer and lymphoma. In an analysis of 500 infliximab-treated patients in Mayo Clinic, serious adverse events were observed in 8.6%, of which 6% was considered to possibly be related to infliximab^[15]. Such events included serious infections, severe infusion reactions, serum sickness-like reactions, drug-induced lupus, cancer, non-Hodgkin's lymphoma and demyelinating process. The infectious complications included fatal sepsis, pneumonia, viral gastroenteritis, abdominal abscesses requiring surgery and histoplasmosis. Five deaths (1%) were observed which were likely or possibly related to infliximab. The reactivation of latent tuberculosis has been reported elsewhere^[16], as a result, it is recommended that all patients be screened for latent tuberculosis before the initiation of this treatment regimen.

Infliximab is a mouse/human chimeric monoclonal antibody of which 25% is mouse peptide sequence. The murine component is ascribed to its immunogenicity, such as infusion-related reactions and serum sickness-like diseases. In such immunological reactions, the formation of antibodies against infliximab, called human anti-chimeric antibodies (HACA) is of particular concern as the presence of HACA is associated with an increased frequency of infusion reactions and the reduction in efficacy^[17]. Concomitant immunosuppressive therapy and premedication with 200 mg of hydrocortisone reduce the frequency of HACA formation^[17,18]. The scheduled infusions in the maintenance therapy have been shown to be associated

with the reduction of the rate of HACA formation^[13]. As a result, the regular infliximab-treatment every 8 wk is likely to be beneficial for CD patients, not only for the maintenance of the remission state but also for the avoidance of infusion reactions.

The efficacy of infliximab for the treatment of UC remains controversial as two randomized controlled trials for steroid-refractory UC resulted in opposite results. A study indicated the benefit of infliximab for UC since 50% (4 of 8 patients) of the infliximab-treated patients showed treatment success, while none of the 3 patients receiving a placebo showed response^[19]. However, this study was terminated prematurely because of a slow enrollment. Another study failed to show any benefit of infliximab over placebo as there was no significant difference between patients who received infliximab and placebo in the remission rates and an improvement in the activity scores^[20]. Recently, two large multicenter randomized trials, ACT1 and ACT2, have been performed. In both trials, 364 patients with active UC were randomized to receive placebo or infliximab in a dose of 5 mg/kg or 10 mg/kg at wk 0, 2, 6, 14 and 22 in ACT1, and at wk 0, 2 and 6 then every 8 wk through wk 46 in ACT2. In both trials, both 5 mg/kg and 10 mg/kg infliximab showed significantly greater percentages in both the induction and maintenance of clinical remission and response, and in mucosal healing than placebo at both wk 8 and 30^[21,22]. In addition, it was also recently demonstrated that infliximab is effective as a rescue therapy to avoid a colectomy or death in severe to moderately severe UC refractory to conventional therapies^[23]. As a result, infliximab thus appears to also be efficacious for the treatment of UC as well as for CD.

CDP571

CDP571 is a "humanized" IgG4 antibody against TNF- α , created by genetic engineering to replace the murine component other than the binding domain with parts of a human IgG4 molecule. The resulting molecule is a chimera of 95% human and 5% mouse residues. The first study of 31 patients with active CD demonstrated that CDP571 5 mg/kg resulted in a greater decrease in the mean CDAI score at wk 2 compared with placebo^[24]. After a promising pilot trial, CDP571 was tested in a placebo-controlled dose-finding trial^[25]. In this study, 169 patients were randomized to receive a single intravenous infusion of either CDP571 in a dose of 10 or 20 mg/kg, or placebo. At wk 2, the clinical response rate was significantly higher in the patients treated with CDP571 (45%) than in those received a placebo (27%). Re-treatment was performed either every 8 wk with a placebo or CDP571 10 mg/kg, or every 12 wk with a placebo or CDP571 10 mg/kg (4 groups). The clinical remission rates at wk 24 in CDP571-treated groups were not significantly different from those of the placebo-treated groups. In a subsequent, randomized, double-blind, placebo-controlled, multicenter study^[26], the efficacy and tolerability of CDP571 in 396 patients with active CD was evaluated. Among the patients treated with CDP571 10 mg/kg every 8 wk, the percentage of patients achieving a clinical response was significantly higher than in those receiving a placebo at wk 2 and 4. However, at wk 28

the difference was not statistically significant. As a result, CDP571 therapy showed a short term benefit in induction therapy, but it is not sufficient to maintain a long term effect. In a post-hoc exploratory analysis of a subgroup of patients with elevated baseline CRP levels, there was a significant difference in the number of patients showing a clinical response at wk 2 (CDP571, 49.5%; placebo, 15.5%), and at all time points from wk 12 to wk 28, thus leaving the possibility that CDP571 is more efficacious in a selected group of patients^[26]. CDP571 failed to show a steroid sparing effect in patients with steroid-dependent CD^[27]. CDP571 was well tolerated even in patients with CD who developed either infusion reactions or delayed-type hypersensitivity reactions to infliximab^[28]. From these results, CDP571 was considered to be safe but not as effective as infliximab for CD and further clinical development of this antibody for the treatment of CD has thus been discontinued.

CDP870

CDP870 is a pegylated Fab fragment of humanized anti-TNF monoclonal antibody. In a placebo-controlled dose-finding study^[29], 292 patients were randomized to receive a subcutaneous dose of CDP870 (100, 200, or 400 mg) or placebo at wk 0, 4, and 8. The group that received CDP870 400 mg showed greater clinical response rates than other groups at all time points. The clinical response rates of the CDP870 400 mg group were significantly higher than those of the placebo treated group at wk 2, 4, 8 and 10. The difference, however, did not reach statistical significance at wk 12. A greater dose separation was evident in the analysis of a patient subgroup with elevated CRP levels. In an exploratory analysis^[30] in 119 patients with increased CRP levels (≥ 10 mg/L), the differences in the clinical response between the 400 mg/dose (53.1%) and placebo (17.9%) were significant at 12 wk. These studies, therefore, indicated that CDP870 may be more effective in patients with elevated CRP levels. CDP870 seems to be safer and less immunogenic than infliximab. The efficacy, however, is likely to be lower than that of infliximab. The question arises whether physicians want to compromise on efficacy in the scope of better long-term safety. Further clinical trials are ongoing.

Etanercept

Etanercept is a genetically engineered fusion protein consisting of two recombinant human TNF p75 receptors linked to an Fc portion of human IgG1 fragment. The subcutaneous injection of etanercept at a dose of 25 mg twice weekly, which is an effective dose for rheumatoid arthritis, is a safe but ineffective dose for the treatment of patients with moderate to severe CD^[31].

Onercept

Onercept, a recombinant, fully human, soluble p55 TNF receptor has showed efficacy to CD in an open-label pilot study ($n = 12$)^[32]. A large, placebo-controlled, dose-finding study has been completed but the data have not yet been published. A press release by Serono (Geneve, Switzerland) revealed that the primary endpoint of this trial was not met.

Adalimumab

Adalimumab is a fully humanized anti-TNF monoclonal IgG1 antibody. This antibody is as efficacious as infliximab for the treatment of rheumatoid arthritis. *In vitro* studies revealed that this antibody is capable of inducing apoptosis in monocytes as infliximab^[33]. As adalimumab does not contain a mouse peptide sequence, it is expected to be less immunogenic and more tolerable than infliximab. Two uncontrolled pilot studies of adalimumab with CD patients who had lost responsiveness or developed intolerance to infliximab^[34,35] showed that subcutaneous adalimumab was well tolerated, thus suggesting a clinical benefit of adalimumab. In a phase 3, multicenter trial for active CD, clinical remission rates of patients who received adalimumab 160 mg at wk 0 and 80 mg at wk 2 was significantly higher than that of placebo at wk 4 (36% *vs* 12%)^[36]. From these results, adalimumab is likely to be efficacious for the treatment of CD and it could thus be an alternative therapy for the patients who either lost responsiveness or developed intolerance to infliximab.

INHIBITION OF OTHER INFLAMMATORY CYTOKINES

Anti-IL-12 p40 antibody

IL-12, a heterodimeric molecule composed of IL-12 p40 and IL-12 p35 subunits, plays a central role in Th1 development. IL-12 is abundantly produced in the gut of CD patients^[2]. In several animal models of Th1-mediated colitis, anti-IL-12 treatment effectively ameliorates intestinal inflammation^[37,38]. IL-12 p40 subunit is also a component of another Th1 cytokine, IL-23, in which p40 forms a heterodimer with p19 subunit. IL-12 p40 is, therefore, a potential target for the treatment of CD in which intestinal inflammation is Th1-mediated. A double-blind, placebo-controlled randomized study of a humanized IgG1 monoclonal antibody against IL-12 p40 (ABT-874) was performed in 79 patients with active CD^[39]. The patients were randomly assigned to receive seven weekly injections of 1 mg/kg anti-IL-12, 3 mg/kg anti-IL-12 or placebo subcutaneously either with or without 4 wk intervals between the first two injections. The patients who received 3 mg/kg anti-IL-12 for 7 wk showed a significantly greater clinical response rate than the patients treated with a placebo (75% *vs* 25%). The rates of remission were also higher in the 3 mg/kg anti-IL-12 group (38%) than in the placebo group (0%) but the difference did not reach statistical significance. The production of IL-12 and other Th1 and proinflammatory cytokines from patients' colonic lamina propria mononuclear cells dramatically decreased after the anti-IL-12 therapy. The most frequent adverse event was a local reaction at the injection site, which was observed with a greater rate in the anti-IL-12-treated group than in the placebo-treated group. Anti-drug antibody was formed in some patients who received anti-IL-12 antibody. No serious side effects requiring the discontinuation of the treatment, due to the anti-IL-12 therapy, were observed. Anti-IL-12 therapy is therefore considered to be a safe and effective treatment for active CD.

MRA (Anti-IL-6 receptor antibody)

IL-6 is one of the major inflammatory cytokines. IL-6 can transduce signals into cells without IL-6 receptor expression when IL-6 binds to soluble IL-6 receptor. The expression of IL-6 and soluble IL-6 receptor increases in patients with active CD^[40,41]. A pilot randomized double-blind placebo-controlled trial of a humanized anti-IL-6 receptor monoclonal antibody, MRA, with active CD was performed^[42]. Thirty-six patients were randomized biweekly to receive either a placebo, 8 mg/kg MRA or MRA/placebo alternately for 12 wk. The clinical remission rate with biweekly MRA was significantly higher than that with placebo (80% *vs* 31%). The acute phase responses such as ESR and CRP levels were rapidly suppressed 2 wk after the MRA injection. The incidence of adverse events was similar in all groups, and thus MRA treatment was generally well tolerated. It is, therefore, likely that anti-IL-6 receptor therapy is beneficial for active CD.

Fontolizumab (Anti-interferon- γ antibody)

Interferon- γ is a key cytokine that enhances the development of a Th1 immune response. Fontolizumab is a humanized monoclonal antibody directed against interferon- γ . A phase 2 study of fontolizumab at intravenous doses of 4 mg/kg or 10 mg/kg in 133 patients with moderate to severe active CD did not demonstrate efficacy at d 28. However, exploratory analyses based on 91 patients who received a second dose of fontolizumab at d 28 did demonstrate efficacy. This effect was most prominent in patients with elevated baseline concentrations of CRP^[43]. An additional phase 2 study of fontolizumab at lower subcutaneous doses of 1.0 mg/kg or 4.0 mg/kg in 196 patients with active CD did not demonstrate efficacy at d 28^[44]. These results indicate that a single dose may not be sufficient to achieve a significant improvement. Further clinical studies of fontolizumab for the induction and maintenance of remission in patients with CD are anticipated.

Anti-IL-2 receptor (CD25) antibodies

Daclizumab: IL-2 is a major T cell growth factor, which is secreted by activated T cells and acts via the high-affinity IL-2 receptor on T cells themselves to promote cell survival and proliferation. The IL-2 receptor α -chain (CD25) is a component of high-affinity IL-2 receptor and it is expressed on activated T cells. Daclizumab is a humanized monoclonal antibody to CD25, which blocks the binding of IL-2 to the IL-2 receptor. An open label pilot study of daclizumab suggested that it was beneficial for patients with active UC^[45]. However, a recent placebo-controlled phase 2 trial of daclizumab at intravenous doses of 1 mg/kg twice with a 4-wk interval or 2 mg/kg every 2 wk for a total of four doses in 159 patients with active UC failed to show any efficacy^[46].

Basiliximab: Basiliximab is a chimeric monoclonal antibody against CD25, which blocks the binding of IL-2 to the IL-2 receptor. Two uncontrolled pilot studies suggested that basiliximab in combination with steroids may be effective for steroid resistant UC^[47,48]. A large random-

ized controlled trial is required to confirm the therapeutic benefit of this compound.

INHIBITION OF ADHESION MOLECULES

Many adhesion molecules play an important role in trafficking leukocytes into the inflamed gut wall and they are up-regulated in both CD and UC^[49,50].

α 4-integrins, predominantly expressed on lymphocytes, usually exist in combination with a β subunit and interact with adhesion molecules expressed on endothelium. α 4 β 1-integrin binds to vascular cellular adhesion molecule 1 (VCAM-1) and α 4 β 7-integrin binds to mucosal addressing cell adhesion molecule 1 (MAdCAM-1). The interaction between α 4 β 7-integrin and MAdCAM-1 is important in mediating lymphocytes homing to the gut mucosa^[51].

Leukocyte function-associated antigen 1 (LFA-1) expressed on leukocytes interacts with intercellular adhesion molecule 1 (ICAM-1), which is constitutively expressed at low levels on vascular endothelial cells and a subset of leukocytes, and they are up-regulated on many cell types in response to proinflammatory mediators^[52].

Natalizumab

Natalizumab, a humanized IgG4 anti- α 4-integrin monoclonal antibody, inhibits both α 4 β 7-integrin/MAdCAM-1 interaction and α 4 β 1/VCAM-1 binding.

In an initial small trial in 30 patients with active CD, a single 3 mg/kg intravenous infusion of natalizumab showed a short term effect in inducing remission at wk 2 and elevated circulating lymphocyte levels after the natalizumab infusion. Therefore, it is suggested that the natalizumab interrupted lymphocyte trafficking into the intestine^[53]. In a large placebo-controlled randomized trial including 248 patients with moderate to severe CD, patients were treated twice at 4 wk intervals with 3 or 6 mg/kg of natalizumab or placebo. A significantly higher number of patients achieved remission at wk 6 only in the 3 + 3 mg/kg natalizumab group compared with the two infusions of placebo group (44% *vs* 27%). The clinical response rates at wk 6 in all treatment groups were significantly higher than that in the placebo-treated group (3 mg/kg natalizumab + placebo: 59%, 3 + 3 mg/kg natalizumab: 71%, 6 + 6 mg/kg natalizumab: 57% and the two infusions of placebo group, 38%)^[54]. A larger phase 3 trial of ENACT-1 in 905 patients with moderate to severe CD failed to show a benefit for three intravenous infusions of 300 mg natalizumab every 4 wk. In a subgroup analysis, however, natalizumab-treated patients with concurrent immunosuppressive therapies, prior anti-TNF- α therapy or elevated CRP levels showed a significant response rate compared with placebo-treated patients^[55]. Three hundred and thirty-nine patients with CD who responded to natalizumab in ENACT-1 were re-randomized to maintenance therapy with natalizumab (300 mg) or a placebo for up to 12 additional monthly infusions. In this maintenance study (ENACT-2), natalizumab demonstrated a significant superiority over the placebo in its ability to sustain both the response and remission at all consecutive time points over a 6-mo period and enabled patients to be successfully withdrawn from

steroids^[56]. In an uncontrolled short term pilot study in 10 patients with active UC, a single 3 mg/kg intravenous infusion of natalizumab showed a short term benefit^[57].

Natalizumab is efficacious in multiple sclerosis (MS) as well^[58]. In MS, α 4 β 1 integrin/VCAM-1 binding appears to be a crucial step because anti- α 4 β 1 integrin antibody prevented the development of experimental autoimmune encephalomyelitis, a model of human MS^[59]. Against these effects of natalizumab in IBD and MS, 3 patients receiving repeated treatment with natalizumab developed JC virus related progressive multifocal leukoencephalopathy (PML)^[60-62]. PML, which almost invariably occurs in patients with AIDS or leukemia or in organ-transplant recipients, is a fatal opportunistic infection of the central nervous system caused by the reactivation of a clinically latent JC polyomavirus infection. Two patients with MS had been receiving the concomitant administration of interferon β -1a^[60,61] and 1 patient with CD had been treated with natalizumab monotherapy^[62]. These observations force us to reconsider both the efficacy and the potential risks associated with an inhibition of lymphocytes trafficking by anti- α 4 integrin therapy.

MLN-02

MLN-02, a humanized anti- α 4 β 7-integrin blocks specifically the α 4 β 7-integrin/MAdCAM-1 interaction.

A randomized placebo-controlled trial in 185 patients with mild to moderately active CD treated with placebo, 0.5 mg/kg MLN-02 or 2.0 mg/kg MLN-02 intravenously on d 1 and 29 demonstrated that on d 57, 2.0 mg/kg MLN-02 showed significantly greater remission rates over the placebo (36.9% *vs* 20.7%). There was no significant difference between the actively treated and placebo-treated groups regarding the clinical response rates. No obvious differences in adverse events were noted among the three groups^[63].

A randomized placebo-controlled trial in 181 patients with moderately active UC treated with placebo, 0.5 mg/kg MLN-02, or 2.0 mg/kg MLN-02 intravenously on d 1 and 29 demonstrated that on d 43 the remission rates were significantly higher in the actively treated groups (0.5 mg/kg: 33%, 2.0 mg/kg: 34%) than in the placebo-treated group (15%). An infusion reaction occurred in one MLN-02-treated patient who developed mild angioedema^[64].

MLN-02 appears to be a generally well-tolerated and effective therapy especially for active UC, but further trials are necessary to confirm the efficacy of MLN-02 therapy for IBD.

Alicaforsen (ISIS 2302)

ISIS 2302 is a 20 base phosphorothioate oligodeoxynucleotide designed to specifically hybridize to the 3'-untranslated region of the human ICAM-1 mRNA. Treatment of ISIS 2302 *in vitro* resulted in a highly specific reduction in ICAM-1 mRNA and, consequently, a marked decrease in ICAM-1 protein expression^[65].

A pilot trial in patients with moderate CD (including 15 patients treated with 13 intravenous infusions of 0.5, 1.0 or 2.0 mg/kg ISIS 2302 *vs* 5 patients with placebo over 26 d) demonstrated a higher remission rate in ISIS 2302-treated group compared with the placebo-treated group on d 33

(47% *vs* 20%)^[66]. However, a placebo-controlled trial in 75 patients with steroid refractory CD failed to demonstrate efficacy was showed that the subcutaneous administration of ISIS 2302 induced only 3% of ISIS 2302-treated patients to clinical remission with complete steroid taper (0% in placebo-treated patients)^[67]. Another larger randomized placebo-controlled trial also failed to show any benefit of ISIS 2302 for active CD^[68]. Two hundred and ninety-nine patients with moderately active, steroid-dependent CD received placebo or ISIS 2302 (2 mg/kg intravenously three times a week) for 2 or 4 wk in mo 1 and 3. There were no differences in the steroid-free remission rates at wk 14 between the ISIS 2302-treated groups (2 wk: 20.2%, 4 wk: 21.2%) and the placebo-treated group (18.8%). However, a subgroup analysis using the area under the curve (AUC) of ISIS 2302 plasma concentration demonstrated an improvement of the clinical response for the highest AUC group (AUC > 65 $\mu\text{g} \times \text{h/mL}$). This suggested that ISIS 2302 may be effective when given in adequate doses. As a result, a dose ranging pilot trial of high dose ISIS 2302 in 22 patients with active CD was conducted. The patients, who were infused with high dose ISIS 2302 (250 mg to 350 mg) intravenously three times a week for 4 wk, showed a 41% remission rate. Five patients, however, withdrew due to infusion-related symptoms^[69].

A randomized placebo-controlled trial in 40 patients with mild to moderately active distal UC treated with 60 mL alicaforsen enema (0.1, 0.5, 2, or 4 mg/mL or placebo) once daily for 28 consecutive days showed a beneficial effect at the highest dose. Four mg/mL alicaforsen enema group showed a significant improvement in the disease activity index on d 29 and mo 3 in comparison to the placebo enema group^[70]. An open-label, uncontrolled study in 12 patients with chronic unremitting pouchitis treated with 240 mg alicaforsen enema nightly for 6 wk demonstrated a significant improvement in the pouchitis disease activity index and an endoscopic mucosal appearance at wk 6^[71].

The most consistently reported side effects of ISIS 2302 in all clinical trials were infusion reactions and a moderate increase in activated partial thromboplastin time.

CONCLUSION

Infliximab has changed the medical treatment of CD dramatically. This agent has been proven to be clearly effective for the induction and maintenance of remission for active CD meanwhile it is also generally well-tolerated. However, there are some patients who fail to respond to infliximab. In addition, some responders either lose responsiveness to infliximab during long-term therapy or develop intolerance to infliximab. The next issue will therefore be how to treat such patients who cannot be treated with infliximab. Adalimumab may be an alternative to infliximab. Anti-IL-12 and anti-IL-6 receptor therapies seem to be promising. The selective blocking of $\alpha 4$ integrin and $\alpha 4\beta 7$ integrin also demonstrated promising results, however, the side effects still need to be fully elucidated. We await the results of further clinical trials to include such novel compounds in the algorithm for the treatment of CD. Infliximab is likely to also be beneficial for UC. In addition, some other agents seem to be effective for the treatment of UC and

further clinical development is also underway. As a result, the management of UC may also dramatically improve in the near future owing to the use of such novel agents. The systematization of novel biological therapies for UC is therefore an issue that needs to be addressed in the future.

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REVIEW

RNAi technology: A Revolutionary tool for the colorectal cancer therapeutics

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Abstract

With the many changes that have taken place in people's diet and lifestyle, colorectal cancer (CRC) has become a global concern. There were approximately 950 000 new cases diagnosed and 500 000 deaths recorded worldwide in 2000. It is the second most common type of cancer in the Western world, and it is the third most common type of digestive tumor in China. It is reported that the morbidity of CRC is 4.08/100 000 for men and 3.30/100 000 for women in China. Despite the rate of improvements in surgery, radiotherapy and chemotherapy, the overall five-year survival is around 50%. Therefore, novel treatment need to be developed in order to add to the therapeutic armamentarium. RNA interference (RNAi) is a sequence-specific post-transcriptional gene silencing mechanism, which is triggered by double-stranded RNA (dsRNA) and causes degradation of mRNA homologous in sequence to the dsRNA. This new approach has been successfully adopted to inhibit virus replication and tumorigenicity. Recent reports have described DNA vector-based strategies for delivery of small interfering RNA (siRNA) into mammalian cells, further expanding the utility of RNAi. With the development of the RNAi technology and deeper understanding of this field, a promising new modality of treatment appeared, which can be used in combination with the existing therapies. We reviewed the proceedings on the actualities and advancement of RNAi technology for colorectal cancer therapeutics.

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Key words: RNAi; Colorectal cancer; Therapeutics

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INTRODUCTION

When viruses infect eukaryotic cells, or when transposons and transgenes are randomly integrated into host genomes, double-stranded RNA (dsRNA) is frequently produced from the foreign genes. Most eukaryotes, including humans, possess an innate cellular immune surveillance system that specifically responds to the presence of dsRNA and activates processes that act post-transcriptionally to silence the expression of the interloping genes^[1-4]. This mechanism is now commonly referred to as RNA interference or RNAi^[5]. During RNAi, long transcripts of dsRNA are rapidly processed into small interfering RNAs (siRNAs), which represent RNA duplexes of specific length and structure that finally guide sequence-specific degradation of mRNAs homologous in sequence to the siRNAs^[6,7]. siRNAs may be the best tools for target validation in biomedical research today and cancer therapeutics, because of their exquisite specificity, efficiency and endurance of gene-specific silencing. Some researches about RNAi technology for colorectal cancer has been reported in recent years. However, these new approaches face serious problems before they can offer a useful adjuvant role for the clinical trials for patients with colorectal cancer.

MECHANISM OF dsRNA INTERFERENCE

The key enzyme required for the processing of long dsRNAs to siRNA duplexes is the RNase III enzyme Dicer, which was characterized in extracts prepared from insect cells, *C. elegans* embryos, mouse cells and mast cells^[8-10]. Dicer contains an N-terminal RNA helicase domain, a Piwi, Argonaute, Zwillie/Pinhead (PAZ) domain^[11], two RNase III domains, and a C-terminal dsRNA-binding motif. The PAZ domain is also present in Argonaute proteins, whose genes represent a poorly characterized family present in dsRNA-responsive organisms. Argonaute1 (AGO1) and Argonaute2 (AGO2), two of the five Argonaute proteins of *D. melanogaster*, appear to be important for forming the mRNA-degrading sequence-specific endonuclease complex, also referred to as the RNA-induced silencing complex (RISC)^[12,13]. Dicer and AGO2 appear to interact in *D. melanogaster* Schneider 2 (S2) cells, probably through their PAZ domains; however, RISC and Dicer activity are separable, and RISC is unable to process dsRNA to siRNAs, suggesting that Dicer is not

a component of RISC. Possibly, the interaction between Dicer and AGO2 facilitates the incorporation of siRNA into RISC^[14]. The endonucleolytic subunit of RISC remains to be identified.

siRNA duplexes produced by the action of Dicer contain 5'-phosphates and free 3'-hydroxyl groups. The central base-paired region is flanked by two-to-three nucleotides of single-stranded 3'-overhangs. The 5' -phosphate termini of siRNAs is essential for guiding mRNA degradation^[15]. Nevertheless, for their application in gene targeting experiments, siRNAs may be used without 5'-phosphate termini because a kinase activity in the cell rapidly phosphorylates the 5' ends of synthetic siRNA duplexes. Under certain circumstances (e.g., injection experiments in *D. melanogaster*), 5' -phosphorylated siRNA duplexes may have slightly enhanced properties as compared with 5'-hydroxyl siRNAs^[16].

In *C. elegans*, introduction of approximately 300 bp dsRNA corresponding to a segment of the targeted gene may also give rise to the phenomenon of transitive RNAi^[17]. Transitive RNAi is characterized by the spread of silencing outside of the region targeted by the initiator dsRNA. Presumably, targeted mRNA serves as template for RNA-dependent RNA polymerase (RdRP) and forms new dsRNA that is processed by Dicer. Thus, secondary siRNAs are generated which may cleave the mRNA out of the region targeted by the ancestral dsRNA.

Until recently, the application of siRNAs in somatic cells was restricted to the delivery of chemically or enzymatically synthesized siRNAs^[18-20], but methods for intracellular expression of small RNA molecules have now been developed. Endogenous delivery is possible by inserting DNA templates for siRNAs into RNA polymerase III (pol III) transcription units, which are based on the sequences of the natural transcription units of the small nuclear RNA U6 or the human RNase P RNA H1. Two approaches are available for expressing siRNAs: (1) The sense and antisense strands constituting the siRNA duplex are transcribed from individual promoters^[20-22], or (2) siRNAs are expressed as fold-back stem-loop structures that give rise to siRNAs after intracellular processing by Dicer^[23,24]. The transfection of cells with plasmids that encode siRNAs, therefore, represents an alternative to direct siRNA transfection. The insertion of siRNA expression cassettes into (retro)viral vectors will also enable the targeting of primary cells refractory to transfection or electroporation of plasmid DNA.

siRNAs AS NOVEL THERAPEUTIC PLATFORM TECHNOLOGY

VEGF is one of the archetypal angiogenic growth factors and has received considerable attention. VEGF is a homodimeric 45 kDa glycoprotein, 5 different isoforms of which are reportedly expressed by endothelial cells. VEGF specifically acts on endothelial cells binding to a growing number of endothelial tyrosine kinase receptors including VEGFR-1 and VEGFR-2. Inhibition of VEGF activity or disabling the function of its receptors has been shown to inhibit both tumor growth and metastasis in a variety

of animal tumor models^[25,26]. Given the different isoforms and their various functions, the development of this RNAi technology and its ability to target specific VEGFs should facilitate both a greater understanding of this field and also the development of improved therapeutics.

Single base pair mutations that alter the function of tumor suppressor genes and oncogenes occur frequently during oncogenesis. The guardian of the genome, p53, is inactivated by point mutation in more than 45%-60% of human colorectal cancers. Synthetic small inhibiting RNAs (siRNAs) are highly sequence-specific reagents and discriminate between single mismatched target RNA sequences, and may represent a new avenue for gene therapy. Martinez LA^[27] demonstrated that a single base difference in siRNAs discriminates between mutant and WT p53 in cells expressing both forms, resulting in the restoration of WT protein function. Therefore, siRNAs may be used to suppress expression of point-mutated genes and provide the basis for selective and personalized antitumor therapy.

The products of bcl-2 genes are involved in the regulation of apoptosis and proliferation and are associated with prognosis in several malignancies, including colorectal adenocarcinoma. A statistically significant inverse association was found between Bcl-2 score and tumor recurrence. It is reported that some researchers use mRNA-cDNA interference for silencing bcl-2 expression in human LNCaP cells^[28]. These findings indicate that a novel gene silencing system may play a useful adjuvant role in the majority of patients with colorectal cancer.

One of the major limitations of current chemotherapy regimes is the bone marrow toxicity associated with these drugs. However, it is well recognized that subpopulations of tumor cells are resistant to particular chemotherapeutic agents and continue to grow in a selective manner in the presence of such drugs. These cells contain specific genes which render them resistant to particular compounds. One such gene is the multiple drug resistance (MDR1) gene which confers resistance to vinca alkaloids (vinblastine, vincristine), anthracyclins (adriamycin, daunorubicin), etoposide and paclitaxel. For reversal of MDR1 gene-dependent multidrug resistance (MDR), two small interfering RNA (siRNA) constructs were designed to inhibit MDR1 expression by RNA interference. Some data indicate that this approach may be applicable to cancer patients as a specific means to reverse tumors with a P-glycoprotein-dependent MDR phenotype back to a drug-sensitive one^[29].

Telomerase is an attractive molecular target toward which to direct cancer therapeutic agents because telomerase activity is present in most malignant cells but undetectable in most normal somatic cells. Kosciolk BA, *et al*^[30] evaluated the ability of siRNA to inhibit telomerase activity in human cancer cells. In their research, human cancer cell lines were transfected with 21 nt double-stranded RNA homologous to either the catalytic subunit of telomerase (human telomerase reverse transcriptase) or its template RNA [human telomerase RNA(hTR)]. Both types of agents reduced telomerase activity in a variety of human cancer cell lines representing both carcinomas and sarcomas. Inhibition was dose-dependent. Telomerase

inhibition by siRNA is notable because telomerase is regarded as restricted to the nucleus, whereas RNA interference is commonly regarded as restricted to the cytoplasm. Their results showed that telomerase activity in human cancer cells can be inhibited by short dsRNAs (siRNAs) targeting telomerase components. Inhibition was shown in a variety of carcinoma cell lines (HCT-15 colon carcinoma, HeLa cervical carcinoma, NCI H23 lung carcinoma, and A431 epidermoid carcinoma).

CRC typically develops over decades and involves multiple genetic events. This has led to the development of a multistep model of colorectal tumorigenesis. In order to identify genes that are important in the development of CRC, RNAi was used to disrupt expression of two of the genes identified by microarray analysis in a colon tumor cell line, HCT116. HCT116 cells were derived from a human colon carcinoma, and showed mutations in β -catenin and K-ras, but possessed wild-type p53^[31]. By examining the growth characteristics of these cells after RNAi both in vivo and in vitro, they hoped to identify targets critical for growth, apoptosis, and/or metastasis. It is clear from these results that siRNA directed against c-myc and survivin lowers the levels of these proteins without affecting the levels of a control protein, β -tubulin. Furthermore, transfection of siRNA oligonucleotides directed against both genes was as effective in reducing protein expression, as were experiments targeting each gene separately. In summary, they have demonstrated that the use of RNAi when coupled with microarray analysis provides an excellent system to define the role of specific genes that are dysregulated in cancer on both the *in vitro* and *in vivo* growth of the tumor^[32].

Somatic changes in CpG dinucleotide methylation occur quite commonly in human cancer cell DNA. Relative to DNA from normal human colonic cells, DNA from human colorectal cancer cells typically displays regional CpG dinucleotide hypermethylation amid global CpG dinucleotide hypomethylation. The role of the maintenance DNA methyltransferase (DNMT1) in the acquisition of such abnormal CpG dinucleotide methylation changes in colorectal cancer cells remains obvious. Some research indicated that human colorectal carcinogenesis is accompanied by a progressive dysregulation of DNMT1 expression and suggest that abnormalities in DNMT1 expression may contribute to the abnormal CpG dinucleotide methylation changes characteristic of human colorectal carcinoma cell DNA^[33]. Recently, there is a report that the controlled knockdown of DNA methyltransferase 1 (DNMT1) in human cancer resulted in growth arrest^[34]. Thus the method allows for a highly controlled approach to gene knockdown.

CONCLUSION

Since biotechnologists have adopted RNAi, it has already earned a place among the major technology platforms. Despite promising data, there are several challenges that need to be faced before RNAi can be used in patients. These include mode of delivery, the precise sequence of the siRNA or shRNA used, and cell type specificity. There are possible toxicities related to silencing of partially

homologous genes or induction of global gene suppression by activating the interferon response. Another potential problem is the inhibition of the function of endogenous miRNAs through competition for the RNAi machinery. Despite these hurdles, RNAi provides the opportunity to pursue an exciting new therapeutic approach to treat colorectal cancer^[35].

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Suppression of bile acid synthesis by thyroid hormone in primary human hepatocytes

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Abstract

AIM: It is known that thyroid hormones alter the bile acid metabolism in humans, however the effect on individual enzymes has been difficult to elucidate. This is mainly due to the lack of human liver cell lines producing bile acids. We used cultures of primary human hepatocytes to study the effects of triiodothyronine (T_3) on bile acid synthesis.

METHODS: Primary hepatocytes were isolated from liver tissue obtained from three different patients undergoing liver resection due to underlying malignancy. The hepatocytes were cultured under serum-free conditions and treated from d 1 to d 5 with culture containing 0.1 - 1000 nmol/L of T_3 . Bile acid formation and mRNA levels of key enzymes were analysed.

RESULTS: The lowest concentration of T_3 decreased cholic acid (CA) formation to 43%-53% of controls and chenodeoxycholic acid (CDCA) to 52%-75% of controls on d 5. The highest dose further decreased CA formation to 16%-48% of controls while CDCA formation remained at 50%-117% of controls. Expression of mRNA levels of cholesterol 7α -hydroxylase (CYP7A1) and sterol 12α -hydroxylase (CYP8B1) dose-dependently decreased. Sterol 27-hydroxylase (CYP27A1) levels also decreased, but not to the same extent.

CONCLUSION: T_3 dose-dependently decreased total bile acid formation in parallel with decreased expression of CYP7A1 and CYP8B1. CA formation is inhibited to a higher degree than CDCA, resulting in a marked decrease in the CA /CDCA ratio.

INTRODUCTION

The effect of thyroid hormone on human bile acid synthesis is still unclear. Several *in vivo* observations have suggested that thyroid hormone influences the bile acid synthesis^[1-3]. However, due to the lack of human *in vitro* tools such as cell lines synthesizing normal bile acids, investigators have not been able to study this at a cellular level. We have previously shown that primary human hepatocytes cultured under serum-free conditions in the presence of matrixgel (EHS) synthesize and conjugate normal bile acids^[4,5]. We also showed that under these conditions they synthesize bile acids with the same capacity and composition as under similar *in vivo* conditions.

In the conversion of cholesterol into bile acids two main pathways are recognized^[6-8]. The neutral pathway is initiated by the 7α -hydroxylation of cholesterol, catalyzed by the microsomal rate-limiting enzyme cholesterol 7α -hydroxylase (CYP7A1). The acidic pathway is initiated by the 27 -hydroxylation of cholesterol, catalyzed by the mitochondrial enzyme sterol 27 -hydroxylase (CYP27A1). In the acidic pathway the microsomal oxysterol 7α -hydroxylase (CYP7B1) mediates the 7α -hydroxylation. Humans synthesize two primary bile acids, cholic acid (CA) and chenodeoxycholic acid (CDCA). CA has a hydroxyl group at the 12^{th} carbon distinguishing it from CDCA. The hydroxyl group is introduced by the microsomal enzyme sterol 12α -hydroxylase (CYP8B1). Therefore, the total amount of intermediates (from either pathway) that undergoes 12α -hydroxylation determines the ratio of CA to CDCA. Both pathways can synthesize CA and CDCA, however the acidic pathway is believed to produce mainly CDCA. The most important mechanism for regulation of bile acid synthesis is the portal flux of bile acids returning to the liver. The loss of bile acids in the intestine (5%-10%) is compensated by *de novo* synthesis.

In this study we examined the effect of different doses

of triiodothyronine (T₃) on bile acid formation and on mRNA levels of *CYP7A1*, *CYP8B1* and *CYP27A1* in cultures of primary human hepatocytes.

MATERIALS AND METHODS

Materials

RPA III™ kit and pTRI-cyclophilin-Human antisense control template were purchased from Ambion Inc. (Huntingdon, UK). ³⁵S-UTP was purchased from Hartmann Analytic GmbH (Braunschweig, Germany). William's medium E with glutamax, gentamycin, penicillin-streptomycin, Dulbecco's phosphate buffered saline, Trizol reagent and Superscript II RT were purchased from Invitrogen (Täby, Sweden). Actrapid (insulin) 100 IU/mL was from Novo Nordisk Pharma (Malmö, Sweden). Collagenase XI-S (Cat. No. C7557), methylthiazolyldiphenyl-tetrazolium bromide (Cat. No. M5655) and triiodothyronine (Cat. No. T6397) were purchased from Sigma-Aldrich (Sweden). Random hexamers (Cat. No. C1181), recombinant RNasin ribonuclease inhibitor (Cat. No. N2511) and RQ DNase I (Cat. No. M6101) were purchased from Promega (Southampton, UK). Taqman universal master mix (Cat. No. 4304437) and human cyclophilin pre-developed assay (now discontinued and replaced by Human PPIA endogenous control (Cat. No. 4310883E) were purchased from Applied Biosystems (Stockholm, Sweden). Dithiothreitol (Cat. No. 708984) was purchased from Roche (Bromma, Sweden).

Isolation of primary human hepatocytes

Human liver tissue was obtained from three different patients undergoing reduction hepatectomy for metastatic cancer to the liver where normal liver tissue was resected due to the localization of the tumor. Patient A was a 62 year old female, patient B a 47 year old male and patient C a 49 year old female.

Approval to use parts of resected human liver specimens for research was given by the ethics committee at Huddinge University Hospital and the patient's informed consent was obtained.

Hepatocytes were isolated by a two-step perfusion technique, utilizing EGTA and collagenase as described in detail by Li *et al.*⁹¹. In brief, liver tissue weighing between 20-150 g was cannulated in 1-2 vessels depending on the size and appearance. The tissue was then perfused with EGTA buffer for about 15 min at a flow rate of 20 mL/min, followed by perfusion with CO₂ (5%) buffered solution containing collagenase XI-S (200 mg /L) at 40°C for approximately 75 min.

Culture conditions

Hepatocytes (3.5×10^6) were cultured under standard conditions in 60 mm culture dishes containing 200 µL EHS matrigel and 3 mL William's E medium with glutamax, supplemented with insulin (2 IU/L), penicillin G sodium (100 U/mL), streptomycin sulphate (100 µg/mL) and gentamycin (85 µg/mL). Cell culture media were collected and renewed everyday. Three 60 millimeter culture dishes each containing 3.5×10^6 hepatocytes were used for each treat-

ment. The cells and media from three dishes were pooled when harvested. The cells were subjected to different concentrations of T₃ (0.1, 1, 10, 100 and 1000 nmol/L) during four days, from d 1 to d 5. On d 5 cell culture media were collected for bile acid analysis and the cells were harvested for RNA isolation and subsequent quantification of specific mRNAs.

Analysis of bile acids in cell culture media

Bile acids in cell culture media were analysed as previously described¹⁴¹. Briefly, 2 mL of the harvested culture medium, together with deuterium labelled cholic acid (D₅) and chenodeoxycholic acid (D₄), was subjected to basic hydrolyzation to remove conjugates and the bile acids were extracted using acidic ether. Bile acids were analysed by gas chromatography-mass spectrometry, as described previously^{11,101}.

Isolation of total RNA

Total RNAs were isolated using Trizol reagent as described by the manufacturer. RNA concentration was determined by spectrophotometry at 260 nm. The purity of the RNA was determined by spectrophotometry at 280 nm and the integrity checked by agarose gel electrophoresis stained with ethidium bromide.

Quantification of CYP7A1 by real time PCR

mRNA levels of CYP7A1 were determined using quantitative single-plex real time PCR. From each group, 40 µg of total RNA was incubated with 5 U of RQ DNase, 5 µL 10 × RQ buffer in a total volume of 50 µL at 37°C for 20 min, at 20°C for 15 min and finally at 70°C for 10 min. From this, 4 µg of DNase treated RNA was used for cDNA synthesis by mixing together with 2 µL random hexamer primers (100 ng/µL), 1 µL dNTP (10 mmol/L of each) in a total volume of 12 µL. After incubation at 65°C for 5 min, 25°C for 10 min and 42°C for 2 min, 2 µL of DTT (0.1 mol/L), 1 µL of RNasin (40 U/µL), 4 µL 5 × first strand buffer and 1 µL superscript II (200 U/µL) were added. The incubation was continued at 42°C for 50 min and at 70°C for 10 min. For real time PCR 3 µL undiluted cDNA/sample was used in triplicates.

Sequences for CYP7A1 primers and probe were kindly provided by Dr. Hermansson, AstraZeneca, Mölndal, Sweden. Reversed primer is: 5'AGA GCA CAG CCC AGG TAT G 3'; forward primer, 5' TGA TTT GGG GGA TTG CTA TA 3' (300 mmol/L); and probe 5' TGG TTC ACC CGT TTG CCT TCT CCT 3' (200 mmol/L). The taqman probe was labelled with FAM and TAMRA. The assays were performed according to ABI user bulletin #2 protocol in a total volume of 25 µL. VIC labelled human cyclophilin pre-developed taqman assay from Applied Biosystems was used for internal control. The assay was performed and analysed using ABI Prism 7700 sequence detector system.

Quantification of CYP8B1 and CYP27A1 by RNase protection assay

Abundance of specific mRNA was quantified using RPA III™. cRNA probes were synthesized using T₃- or T₇-polymerase and labelled with ³⁵S-UTP. Cyclophilin was

used as an internal standard and gave a protected fragment size of 103 bp.

The protected fragment size of the probe for CYP27A1 was 297 bp and for CYP8B1 442 bp. The assay was performed according to the manufacturer's protocol. In brief, 10-15 µg of total RNA was co-precipitated in 2.5 volumes of ethanol and 0.5 mol/L NH₄OAc with about 80 000 cpm of each probe at -20°C for 15 min. Following centrifugation at 15 000 rpm for 15 min the pellets were dissolved in 10 µL hybridisation buffer, heated to 95°C for 3 min and incubated at 46°C overnight. The hybridised RNA samples were then treated with RNase A and RNase A/T1 diluted 1:50 in RNase solution at 37°C and inactivated after 30 min by adding inactivation/precipitation solution. Following precipitation at -20°C for 15 min the samples were centrifuged at 15 000 r/min for 15 min and the pellets were air dried and dissolved in 10 µL of loading buffer.

The protected fragments were separated on a denaturing 5% polyacrylamide urea gel and detected and quantified using a Fuji BAS 1800 Phospho-imager.

Statistical analysis

The significance of differences between groups for the mRNA analysis was tested by a one-way ANOVA followed by a post-hoc test. To stabilize the variances, data were logarithmically transformed. Due to variances between patients, statistical analysis of the bile acid formation could not be performed.

RESULTS

Purity of hepatocyte preparations

We have analyzed the purity of the preparation of hepatocytes that we plated. Following the final low speed centrifugation step the cell pellet was resuspended in plating media and samples of the cell preparation were spotted onto microscopic glass slides, air dried and fixed in formalin. Preparations from 2 separate human cases were analyzed. Duplicate slides were exposed to antibodies for low molecular weight cytokeratins (AE1/AE3), albumin or hepatocyte specific antibody HEPR. The results showed that 93% of the cells in the preparation reacted with antibodies to cytokeratin, albumin and HEPR, indicating that 93% of the cells plated on culture dishes were epithelial (cytokeratin positive) and parenchymal hepatocytes (HEPR and albumin positive). Most of the remaining cells were small and appeared to be hematopoietic. Hematopoietic cells would not be expected to attach to culture dishes and would be removed by media changes.

Patient A

The cells were treated for 4 d (from d 1 to d 5) with different concentrations of T₃. The cell culture media were changed daily and collected on d 5 of culture after 24 h incubation. In control cells the total bile acid formation was 1515 ng/mL of culture media (920 ng CA and 595 ng CDCA per mL) and the CA/CDCA ratio 1.5. Treatment with 0.1 nmol/L of T₃ decreased total formation by

Table 1 Formation of CA and CDCA in primary human hepatocytes obtained from 3 patients

Patient	Addition of T ₃	CA	CDCA	Ratio
		ng/mL	Medium/24 h	CA/CDCA
A	Control	920	595	1.5
	0.1 nmol/L	400	310	1.3
	1 nmol/L	375	375	1.0
	10 nmol/L	340	390	0.9
	100 nmol/L	250	285	0.9
	1 µmol/L	245	295	0.8
B	Control	280	60	4.7
	0.1 nmol/L	135	45	3.0
	1 nmol/L	95	70	1.4
	10 nmol/L	55	60	0.9
	100 nmol/L	45	65	0.7
	1 µmol/L	45	70	0.6
C	Control	805	502	1.6
	0.1 nmol/L	430	345	1.2
	1 nmol/L	477	392	1.2
	10 nmol/L	395	425	0.9
	100 nmol/L	372	405	0.9
	1 µmol/L	382	420	0.9

The cells were cultured in a serum-free medium and treated with different concentrations of T₃ for 5 d. Bile acids were measured in the cell-free medium. CA: Cholic acid; CDCA: Chenodeoxycholic acid.

53% to 710 ng/mL and lowered the CA/CDCA ratio to 1.3 (Table 1). Higher doses of T₃ decreased bile acid formation even further in a dose dependent manner, down to 36% of control with a CA/CDCA ratio of 0.8 for the highest dose, 1000 nmol/L.

Expression of CYP7A1 mRNA levels was decreased by all doses of T₃ tested. Addition of 0.1 nmol/L of T₃ decreased CYP7A1 mRNA levels from 4.45 to 0.54 (arbitrary value) (12% of control), 10 nmol/L to 0.44 and 1000 nmol/L further down to 0.34 (8% of control). Two of the measure points were lost due to human error (Figure 1).

Similarly, expression of CYP8B1 mRNA decreased with T₃ treatment. Addition of 0.1 nmol/L of T₃ decreased CYP8B1 from 0.226 to 0.056 (arbitrary value) (12% of control), 10 nmol/L to 0.028 and 1000 nmol/L to 0.017 (8% of control) (Figure 1).

Also CYP27A1 mRNA levels decreased with T₃ treatment. Addition of 0.1 nmol/L lowered CYP27A1 levels from 1.050 to 0.408 (arbitrary value) (39% of control), 10 nmol/L to 0.373 and 1000 nmol/L to 0.334 (32% of control) (Figure 1).

Patient B

The bile acid formation in hepatocytes from patient B was much lower than in cells from patients A and C. In control cells the total bile acid formation was 340 ng/mL of culture media (280 ng CA and 60 ng CDCA per mL) and the CA/CDCA ratio was 4.7. Treatment with 0.1 nmol/L of T₃ decreased total formation by 47% to 180 ng/mL, and lowered CA/CDCA ratio to 3.0 (Table 1). Higher doses of T₃ decreased bile acid formation even further down to 34% of control and CA/CDCA ratio to 0.6 for

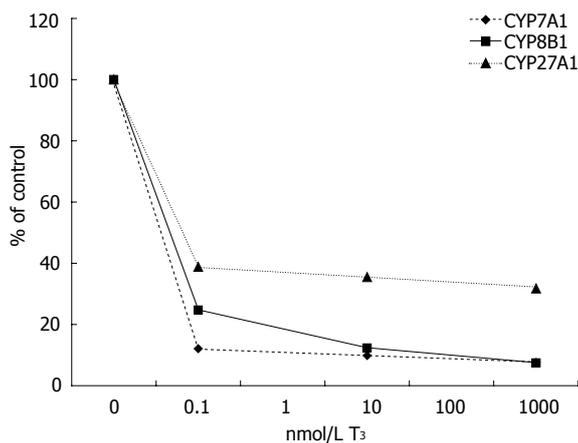


Figure 1 Patient A, effect of T₃ on CYP7A1, CYP8B1 and CYP27A1 mRNA levels normalized to cyclophilin and expressed as % of control.

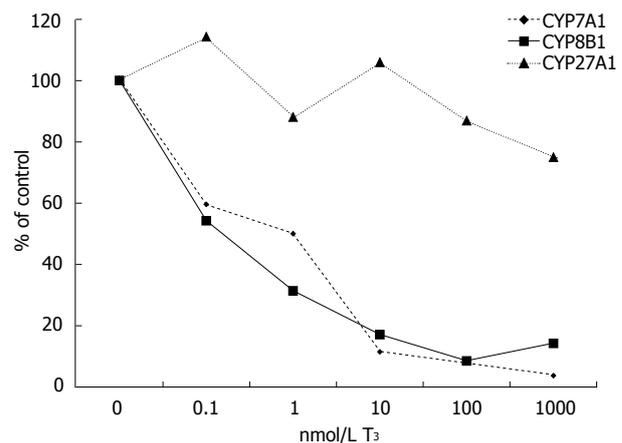


Figure 2 Patient B, effect of T₃ on CYP7A1, CYP8B1 and CYP27A1 mRNA levels normalized to cyclophilin and expressed as % of control.

the highest dose, 1000 nmol/L.

Expression of CYP7A1 mRNA levels was decreased by all doses of T₃ tested but not to the same extent as in cells from patient A. Addition of 0.1 nmol/L of T₃ decreased CYP7A1 mRNA levels from 0.745 to 0.444 (arbitrary value) (60% of control), 1 nmol/L to 0.373, 10 nmol/L to 0.086, 100 nmol/L to 0.063 and 1000 nmol/L further down to 0.028 (4% of control) (Figure 2).

Similarly, expression of CYP8B1 decreased with T₃ treatment. Addition of 0.1 nmol/L of T₃ decreased CYP8B1 mRNA levels from 0.175 to 0.095 (arbitrary value) (54% of control), 1 nmol/L to 0.055, 10 nmol/L to 0.030, 100 nmol/L to 0.015 and 1000 nmol/L further down to 0.025 (14% of control) (Figure 2).

T₃ had a modest effect on the expression of CYP27A1 in patient B. Addition of 0.1 nmol/L of T₃ changed CYP27A1 mRNA levels from 0.420 to 0.480 (arbitrary value) (114% of control), 1 nmol/L to 0.370, 10 nmol/L to 0.445, 100 nmol/L to 0.365 and 1000 nmol/L down to 0.315 (75% of control) (Figure 2).

Patient C

In control cells total bile acid formation was 1308 ng/mL of culture media (805 ng CA and 502 ng CDCA per mL) and the CA/CDCA ratio was 1.6. Treatment with 0.1 nmol/L of T₃ decreased the total formation by 41% to 775 ng/mL and lowered the CA/CDCA ratio to 1.2 (Table 1). Higher doses of T₃ decreased bile acid formation to 61% of control and lowered the CA/CDCA further down to 0.9 for the highest dose, 1000 nmol/L.

Expression of CYP7A1 mRNA levels was decreased by all doses of T₃ tested, but not as strongly as in patients A and B. Addition of 0.1 nmol/L of T₃ decreased CYP7A1 mRNA levels from 3.15 to 1.64 (arbitrary value) (52% of control), 1 nmol/L to 2.06, 10 nmol/L to 1.06, 100 nmol/L to 0.81 and 1000 nmol/L further down to 0.65 (21% of control) (Figure 3).

Similarly, expression of CYP8B1 mRNA decreased with T₃ treatment. Addition of 0.1 nmol/L of T₃ decreased CYP8B1 mRNA levels from 0.093 to 0.070 (arbitrary value) (75% of control), 1 nmol/L to 0.040, 10 nmol/L to 0.050, 100 nmol/L and 1000 nmol/L both to

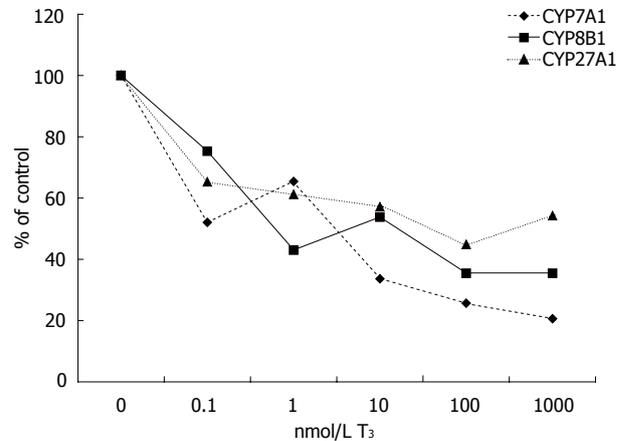


Figure 3 Patient C, effect of T₃ on CYP7A1, CYP8B1 and CYP27A1 mRNA levels normalized to cyclophilin and expressed as % of control.

0.033 (35% of control) (Figure 3).

Also CYP27A1 mRNA levels decreased with T₃ treatment. Addition of 0.1 nmol/L lowered CYP27A1 levels from 0.245 to 0.160 (arbitrary value) (65% of control), 1 nmol/L to 0.150, 10 nmol/L to 0.140, 100 nmol/L to 0.110 and 1000 nmol/L to 0.133 (54% of control) (Figure 3).

Following logarithmical transformation, statistical analysis of mRNA levels from all three patients showed that the highest dose (1000 nmol/L) of T₃ significantly decreased CYP7A1 mRNA levels ($P < 0.05$). CYP8B1 mRNA levels was significantly decreased by 10 and 100 nmol/L of T₃. Treatment with T₃ did not significantly decrease CYP27A1 mRNA levels.

DISCUSSION

In accordance with our previous studies^[4,5,11,12], CA and CDCA, constituted more than 95% of total sterols and the ratio between the two bile acids averaged 2.6.

T₃ dose-dependently decreased formation of bile acids to 44% of controls. The reduction of CA formation was greater than the decline in CDCA formation and

resulted in a marked decrease in the CA/CDCA ratio. These results are in reasonable agreement with previous *in vivo* studies. Angelin *et al*^[13] studied bile acid metabolism in hypothyroid patients before and during replacement therapy to euthyroid state and found that CDCA synthesis was stimulated by approximately 40%, whereas CA did not change with hormone treatment. However, the relative concentration of deoxycholic acid (DCA) in bile decreased from 30% to 19% and CDCA concomitantly increased with hormone therapy. This was confirmed by Kosuge *et al*^[2] who concluded that the characteristic effect of thyroid hormone on serum bile acid composition in man is the shift from the "family" of cholic to that of CDCA by showing that DCA is the most prominent bile acid in hypothyroid patients and that CDCA is the most prominent bile acid in hyperthyroid patients. Paulezki *et al*^[3] studied bile acid metabolism in hyperthyroid patients before and during treatment to euthyroid state and showed that thyroid hormone excess caused a 34% reduction in CA synthesis with no apparent change of CDCA synthesis and a 20% decrease in total bile acid synthesis. The authors suggested that thyroid hormone inhibited hepatic 12 α -hydroxylation.

In contrast to this, Sauter *et al* failed to show any effect of T₃ treatment on the serum levels of the bile acid intermediate 7 α -hydroxy-4-cholestene-3-one (C4) (believed to reflect CYP7A1 activity *in vivo*) in hypo- and hyperthyroidism before and after treatment to euthyroid state^[14]. A recent study by Galman *et al* showed that there are marked diurnal changes of C4 levels during the day^[15]. The authors could also see a weak but significant correlation of C4 with cholesterol and therefore corrected C4 levels for cholesterol. These findings make it difficult to evaluate the results of the C4 levels of the mentioned study. Also, the fact that C4 is the main substrate for CYP8B1 and that one study suggested that CYP8B1 might be inhibited by thyroid hormone needs to be considered.

The mRNA levels of the rate-limiting enzyme CYP7A1 in the neutral pathway were also dose-dependently decreased by T₃. Our data support the human promoter studies by Wang *et al* who found that thyroid hormone repressed CYP7A1 activity^[16]. Furthermore, Drover *et al* found a distinct thyroid hormone response element that mediates T₃ repression in the human CYP7A1 promoter^[17]. This is in contrast to the study in rats where Crestani *et al* failed to demonstrate an effect of thyroid hormone on the *Cyp7a1* promoter activity^[18]. However addition of 1 μ mol/L of T₄ to primary rat hepatocytes increased the transcriptional activity of *Cyp7a1* by 3.7 fold but did not alter steady state mRNA levels^[19].

In the present study we did not measure the enzyme activity of CYP7A1 because of lack of material, but since the effects on the mRNA levels were in agreement with the effects on the bile acid formation we believed that it would be unlikely that T₃ would have an opposite effect on the enzyme activity.

Similar to CYP7A1, the CYP8B1 mRNA levels were also dose-dependently decreased by T₃. Although previous *in vivo* data pointed towards an effect on CYP8B1, this is to our knowledge the first report of the effects of T₃ on human CYP8B1 mRNA levels. In rats however, T₃ has

been shown to suppress both Cyp8b1 activity and mRNA levels^[20].

Treatment with T₃ also decreased CYP27A1 levels, but to a much lower degree than CYP7A1 and CYP8B1. This finding is in line with the contention that CYP27A1 is regulated to a lower degree than CYP7A1 in humans, as has been shown in connection with bile acid mediated feedback regulation^[21]. According to a previous study by Stravitz *et al*^[22] *Cyp27a1* is not regulated by thyroid hormone at all in rat.

In a previous study^[4] we investigated the effect of the combination of T₃ and dexamethasone on bile acid synthesis in rat *vs* human hepatocytes. Since it was previously shown that T₃ and dexamethasone had a synergistic effect on *Cyp7a1* mRNA levels in primary rat hepatocytes^[19] and since neither T₃ nor dexamethasone alone had any effect in rat hepatocytes, we did not focus on the effect of T₃ or dexamethasone alone in primary human hepatocytes. However, we could see that T₃ and dexamethasone alone tended to decrease bile acid formation but had no consistent effect on CYP7A1 mRNA levels (data not shown). The results of the present study and the fact that combination of T₃ and dexamethasone did not change human bile acid formation or CYP7A1 mRNA levels in our prior study, suggest that dexamethasone might have an effect on human bile acid synthesis. This is very interesting and needs to be elucidated in future studies.

The concentrations of T₃ used in this study ranged from 0.1 nmol/L to 1000 nmol/L. The highest dose was included because this is a dose commonly used in combination with dexamethasone in cultures of primary rat hepatocytes. Reduction of methylthiazolyldiphenylterazolium bromide (MTT) showed no signs of cytotoxicity in the dose range used here (data not shown). It should be emphasized that the most dramatic effects were seen with the lowest T₃ concentration, supporting that the effects observed are physiological.

In conclusion, it has been shown that T₃ dose-dependently decreases bile acid formation and expression levels of CYP7A1 and CYP8B1 mRNAs in primary human hepatocytes. CYP27A1 is also decreased, but to a lower degree. CA formation is inhibited to a higher degree than CDCA formation, resulting in a marked decrease in the CA/CDCA ratio.

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Cross-species hybridization of woodchuck hepatitis virus-induced hepatocellular carcinoma using human oligonucleotide microarrays

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Abstract

AIM: To demonstrate the feasibility of using woodchuck samples on human microarrays, to provide insight into pathways involving positron emission tomography (PET) imaging tracers and to identify genes that could be potential molecular imaging targets for woodchuck hepatocellular carcinoma.

METHODS: Labeled cRNA from woodchuck tissue samples were hybridized to Affymetrix U133 plus 2.0 GeneChips[®]. Ten genes were selected for validation using quantitative RT-PCR and literature review was made.

RESULTS: Testis enhanced gene transcript (BAX Inhibitor 1), alpha-fetoprotein, isocitrate dehydrogenase 3 (NAD⁺) beta, acetyl-CoA synthetase 2, carnitine palmitoyltransferase 2, and N-myc2 were up-regulated and spermidine/spermine N1-acetyltransferase was down-regulated in the woodchuck HCC. We also found previously published results supporting 8 of the 10 most up-regulated genes and all 10 of the 10 most down-regulated genes.

CONCLUSION: Many of our microarray results were validated using RT-PCR or literature search. Hence, we believe that woodchuck HCC and non-cancerous liver samples can be used on human microarrays to yield meaningful results.

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INTRODUCTION

Patients with chronic hepatitis originating from infection with hepatitis B virus (HBV) are among those with the highest risk for hepatocellular carcinoma (HCC). The role of HBV in hepatocarcinogenesis has been studied by many investigators, but still not entirely understood. The first case of hepatocellular adenomas in a woodchuck was reported by Fox in 1912^[1]. The model of hepatitis-induced HCC was later described by Summers in 1978^[2] and is now an accepted animal model for studying HBV-induced HCC^[3]. We have performed PET imaging with various positron-emitting radionuclide-labeled imaging tracers involved in the woodchuck hepatitis virus (WHV)-induced HCC animal model (details of the imaging study will be reported separately). Early studies in this project include investigating the genetic basis of pathways involving the tracers and looking for new genetic targets for molecular imaging.

In the past decade, microarray experiments have paved the way for thorough studies of gene expression. To date there has been no commercially available microarray to study gene expression in the woodchuck largely because the genome of this species has not been sequenced and it is not among the common animal models. Cross-species hybridization analysis is one of the methods to investigate genetic regulation of unusual species. Some authors have succeeded in using porcine samples on human Affymetrix GeneChips[®]^[4,5]. One group has demonstrated the feasibility of using normal woodchuck samples on commercially available human nylon membrane arrays that have since been discontinued^[6].

To the best of our knowledge, there have been no studies that used woodchuck samples on human

Table 1 RT-PCR primer and probe sequences for select genes

Target gene	Forward primer sequence	Reverse primer sequence	Reporter probe sequence
Testis enhanced gene transcript (BAX inhibitor 1) (TEGT)	GCCACCTGTAATGTGTGCAAAG	ACTTCCCAGTTTGACTTCCTCTC	CTCCACCCCTCATGTCC
Acetyl-CoA synthetase 2 (thiokinase) (ACAS2)	TGCTGAGGACCCACTCTTCAT	AGCATGTAACCGCCAACGT	CCACAGGCAAACCCA
Alpha-fetoprotein (AFP)	AGGCTGTTCATTGCAGATTTCTCT	GGACCCTCTTCTGCAAAGCA	CTGGCAGCATGTCTCC
C-myc (CMYC)	CAGAAGGTCAGAATCGGTGTCA	AGGACCAGTGGGCTGTGA	CCACAGCAAACCTC
Hexokinase (glucokinase regulatory protein) (GCKR)	ATGCTGCAGCGTTCCT	GGATCGCTTGGAGGAGACTCT	AAGGCCCGATGCATTG
Isocitrate dehydrogenase 3 (NAD+) beta (IDH3B)	TCTCAGCGGATTGCAAAGTTTG	CTGTGGACAGCTGTGACCTT	CCCGCCCTTCTTG
Fatty-acid-Coenzyme A ligase, very long-chain 1 (FACVL1)	GCGGGATGACACAGCAAAA	TCTTCTGGAAATGTGAGTTATCCTTCTG	TCTGAAAATCTGAATATCCC
Spermidine/spermine N1-acetyltransferase (SSAT)	TTTCATGCAACACTTGGTCTCTCT	CCTGGACTCCGGAAGGTAAC	CCCTACCCAATCCAG
Carnitine palmitoyltransferase II (CPT2)	TGCCTATTCCTCAAACCTGAAGACA	TCTTCTGAACTGGCCATCATT	TCAATGCACAGAAACCT
N-myc 2 (NMYC2)	GGACGCGCTGAGTGGAT	GCTCTCGTTCGGCTCCAA	TTCCAGGAGACCCC

oligonucleotide arrays to explore differential gene expression in non-cancerous woodchuck liver versus HCC. In this study, we analyzed the woodchuck gene expression using human Affymetrix GeneChips[®] with validation of differential expression of selected genes with quantitative RT-PCR and literature search.

MATERIALS AND METHODS

RNA isolation

All animals received humane care and the study protocol complies with the institutional guidelines. Woodchucks were euthanized after PET imaging and the livers were immediately removed from the animal. A pathologist separated non-cancerous liver and HCC. Tissues were snap-frozen in liquid nitrogen and stored at -80°C. RNA was extracted with RNeasy Midi Kit from Qiagen (Valencia, CA) according to the recommended protocol. An RNA integrity number (RIN) was found using a Bioanalyzer 2100 from Agilent (Palo Alto, CA).

Preparation of cRNA and microarray hybridization

RNA analyses were performed in the Gene Expression Array Core Facility at Case Western Reserve University. cRNA was prepared and hybridized to Affymetrix Human U133 plus 2.0 GeneChips[®] (Santa Clara, CA) according to the manufacturer's instructions. Microarray results were delivered in the form of cab files.

Microarray data analysis

Two sample *t* tests of quality control data were performed using R 2.0.1 statistical software. Hybridization results were analyzed using GeneChip[®] Operating Software (GCOS) 1.2 from Affymetrix (Santa Clara, CA). All microarrays were scaled to the same average signal intensity so that they could be compared to one another. Microsoft Excel and Access (Redmond, WA) were used to screen the data for significant results. Significantly changed genes were found using a modified version of the criteria outlined

for a Comparison Analysis in the GeneChip[®] Expression Analysis: Data Analysis Fundamentals Manual from Affymetrix (Santa Clara, CA). The only criteria modified was to include signal fold changes for genes that were up-regulated by at least 1.5 or down-regulated by at least -1.5 in the HCC compared with the non-cancerous tissues.

RT-PCR

Available sequence information for woodchuck genes was found in the nucleotide database in the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov). These sequences were put into Applied Biosystems (Foster City, CA) for determination and production of optimal Custom Taqman[®] Gene Expression Assays. The Taqman[®] primer and probe sequences for our genes of interest are listed in Table 1. The Taqman[®] Assays and total RNA were given to the Gene Expression Array Core Facility. Assays were run in triplicate in a 15 µL reaction volume in a 384-well plate using a PRISM[®] 7900HT Sequence Detection System from Applied Biosystems (Foster City, CA). The RT-PCR assay consisted of a 2-minute incubation at 50°C, a 10-minute incubation at 95°C followed by 40 cycles of a 15-second incubation at 95°C, and then a 1-minute incubation at 60°C. Results were provided to us as values relative to a baseline sample. HCC was compared with the non-cancerous baseline sample from each animal, and > 2.5 or < 2.5 fold gene expression was considered up- or down-regulated, respectively.

RESULTS

Quality control

The first step in determining the usefulness of the results from a microarray study is to examine the quality control data. Table 2 shows some of RNA and microarray quality control data that we examined in this study. Two sample *t* tests were performed to see if there were differences between normal liver samples and HCC samples in any of the categories listed in Table 2. The "Percentage of

Table 2 Quality control data for cross-species hybridization

Samples	RNA integrity number (max = 10.0)	Average background	Noise	Percentage of genes called present	Scaling factor (scaled to 15)
W904Nb	7.5	33.750	1.00	9.0	2.959
W361N	7.8	34.170	0.97	9.5	2.970
W380N	9.7	32.089	0.93	9.9	2.955
W904T	9.8	36.880	1.07	10.3	2.206
W361T	9.2	35.720	1.05	10.5	2.355
W380T	9.9	31.610	0.94	10.8	2.463

Genes Called Present” and the “Scaling Factor” showed a statistically significant difference ($P < 0.05$) between the normal and the tumor samples. Housekeeping control and spiked control genes were qualitatively examined to have similar signal values and similar 3'-to-5' ratios across all of the samples.

Significantly changed genes

A number of the genes was found to be significantly changed in the HCC tissues compared with the normal portion of the liver from the same animal (Table 3). Sixty-six genes were found significantly changed in all three animals and 286 genes changed in 2 out of the 3 animals. Some genes with multiple probes on the microarray were also markedly changed. Of the 18 gene probes that were down-regulated in all 3 tumors, 5 presented metallothioneins, 3 for early growth response 1, and 2 for spermidine/spermine N1-acetyltransferase. Five genes were up-regulated in 2 probes in all 3 tumors: H2A histone family member Z, F-box protein 9, a disintegrin and metalloproteinase domain 10, butyrate-induced transcript 1, and ribophorin II.

We studied further into the 10 most up- and down-regulated genes that were common to all three HCC versus normal liver sample pairs (Table 4). Eight of the top 10 up-regulated genes found previously in literature are in agreement with our findings^[7-14]. We were unable to find any publication describing the up-regulation in any cancer for 2 of these genes, one of which was an open reading frame, but found published data supporting all of the 10 most down-regulated genes^[15-21].

One group used normal human and normal woodchuck liver samples on human nylon membrane arrays^[6]. To compare our results to theirs, we looked into 48 genes which were found to be commonly expressed in both normal human and normal woodchuck livers. We used 2 normal woodchuck liver samples from uninfected woodchucks on the Affymetrix human U133 plus 2.0 GeneChip[®] and found 21 of the 48 previously reported genes to be expressed. We were unable to find any probes on the GeneChip[®] for 3 of the 48 genes. We also explored the normal portion of the liver from 3 woodchucks with HCC and found 22 of the 48 genes were present in all 3 animals, 26 were present in 2 animals and 34 were present in at least 1 animal. Eighteen genes were present in both the normal liver from an uninfected woodchuck and the non-cancerous liver samples from all 3 woodchucks with HCC.

Table 3 Significantly changed genes

Sample pair (tumor vs normal)	Number of up-regulated genes ¹	Number of down-regulated genes ¹	Total number of changed genes
W904	596	205	801
W361	359	242	601
W380	215	81	296
W904, W361	142	47	189
W904, W380	66	3	69
W361, W380	19	9	28
W904, W361, W380	48	18	66

¹The data is presented as HCC vs normal portion of the liver sample where the normal sample is the baseline.

RT-PCR

Ten genes were selected for RT-PCR analysis. Two genes were selected to validate the microarray analysis because the microarray analyses demonstrated they were significantly changed (TEGT and SSAT). RT-PCR was also performed on other genes due to their role in metabolic pathways and hepatocellular carcinogenesis. The RT-PCR results along with the corresponding microarray results are shown in Table 5. It should be noted that the terms “no change” and “undetermined” are not the same. A sample with no change shows similar expression in the normal and tumor samples, whereas the undetermined means that expression could not be measured in either the normal or tumor sample using RT-PCR.

DISCUSSION

PET imaging allows clinicians to view *in vivo* distribution and uptake of a radiolabeled compound, or imaging tracer. It is a useful diagnostic tool when a tracer is used that is highly specific to its target. We investigated the performance of various tracers and their metabolism regulated at multiple levels in the woodchuck model of hepatitis virus-induced HCC. We conducted a microarray study to explore the transcriptional regulation of interesting genes.

There is a logical rationale to explain the disparity in quality control data between normal and tumor samples. In the normal tissue, there were fewer genes called “Present” and a larger scaling factor as shown in Table 2. More genes were up-regulated in the tumors than were down-regulated. Therefore, more genes were expressed in the tumors compared with the non-cancerous portion of the liver, hence the larger ‘Percentage of Genes Called Present’. The fact that fewer genes were called ‘Present’ in the non-cancerous samples is the likely reason for the lower overall average signal intensity, which would also mean that a larger scaling factor is needed to reach a target value compared with the tumor samples.

To determine if our microarray results were consistent with other studies, we compared our results using normal woodchuck liver samples from 2 uninfected animals on Affymetrix U133 plus 2.0 GeneChips[®] to the results of another group that used similar uninfected woodchuck

Table 4 Top 10 up- and down-regulated genes in common on all three tumor/normal sample pairs

Fold change ¹	Gene title	Gene symbol	Location	Process	Agreement
14.3294	Stearoyl-CoA desaturase (delta-9-desaturase)	SCD	10q23-q24	Fatty Acid Biosynthesis	[7]
11.6297	ELOVL family member 6, elongation of long chain fatty acids (FEN1/Elo2, SUR4/Elo3-like, yeast)	ELOVL6	4q25	Fatty Acid Biosynthesis	[8]
7.7381	Chromosome 9 open reading frame 41	C9orf41	9q21.13		none
5.1036	Cytochrome P450, family 51, subfamily A, polypeptide 1	CYP51A1	7q21.2-q21.3	Electron Transport/ Cholesterol Biosynthesis	[9]
4.1746	H2A histone family, member Z	H2AFZ	4q24	DNA Replication	[10]
3.4227	H2A histone family, member Z	H2AFZ	4q24	DNA Replication	[10]
3.3977	TATA box-binding protein-like protein 1, TBP-like 1	TBPL1	6q22.1-q22.3	Transcription	none
2.9307	Diacylglycerol O-acyltransferase homolog 2 (mouse)	DGAT2	11q13.5	Triacylglycerol biosynthesis	[11]
2.8134	ARP2 actin-related protein 2 homolog (yeast)	ACTR2	2p14	Protein Binding/Structural Molecule Activity	[12,13]
2.8134	Calcium binding protein P22	CHP	15q13.3	Potassium Ion Transport/Small GTPase Mediated Signal Transduction	[14]
-3.6151	Metallothionein 1H	MT1H	16q13	Metal Ion Binding	[15,16]
-3.6353	Metallothionein 2A	MT2A	16q13	Metal Ion Binding	[15,16]
-3.9071	Metallothionein 1X	MT1X	16q13	Metal Ion Binding	[15,17]
-4.1870	EST similar to early growth response 1				[18,19]
-4.5774	Metallothionein 1F (functional)	MT1F	16q13	Metal Ion Binding	[15,16]
-4.9625	Early growth response 1	EGR1	5q31.1	Regulation of Transcription	[18,19]
-5.5956	Metallothionein 1F (functional)	MT1F	16q13	Metal Ion Binding	[15,16]
-5.7266	Sprouty homolog 2 (Drosophila)	SPRY2	13q31.1	Cell-Cell Signaling/ Development/ Organogenesis/ Regulation of Signal Transduction	[20]
-10.3842	Early growth response 1	EGR1	5q31.1	Regulation of Transcription	[18,19]
-24.2139	Insulin-like growth factor binding protein 2, 36 kDa	IGFBP2	2q33-q34	Regulation of Cell Growth	[21]

¹This is the average fold change for all 3 HCC *vs* normal sample pairs.

Table 5 RT-PCR and microarray results

Gene	Process	W904		W361		W380	
		RT-PCR	Microarray	RT-PCR	Microarray	RT-PCR	Microarray
Testis enhanced gene transcript (BAX inhibitor 1) (TEGT)	Negative regulation of apoptosis	No change	Up	Up	Up	Up	Up
Spermidine/spermine N1-acetyltransferase (SSAT)	Polyamine metabolism	Down	Down	Down	Down	No change	Down
Acetyl-CoA synthetase 2 (thiokinase) (ACAS2) ¹	Metabolism, lipid biosynthesis	Up	Up	Up	Up	Up	No change
Alpha-fetoprotein (AFP)	Immune response, transport	Up	No change	Up	No change	Up	Up
Iso citrate dehydrogenase 3 (NAD+) beta (IDH3B)	Tricarboxylic acid cycle	Up	Up	Undetermined	No change	Up	Up
Carnitine palmitoyltransferase II (CPT2)	Lipid metabolism, fatty acid metabolism, transport	Up	No change	Up	No change	No change	No change
N-myc 2 (NMYC2)	Unknown	Undetermined	N/A	Up	N/A	Undetermined	N/A
C-myc (CMYC) ²	Regulation of transcription, DNA-dependent	Up	Up	Down	Up	No change	No change
Fatty-acid-Coenzyme A ligase, very long-chain 1 (FACVL1)	Lipid metabolism, fatty acid metabolism	Down	Down	Undetermined	Down	Up	No change
Hexokinase (GCKR) ³	Carbohydrate metabolism	Undetermined	No change	Undetermined	Up	Up	No change

¹RT-PCR results are for ACAS2, microarray results are for a hypothetical protein similar to ACAS2. ²RT-PCR results are for c-myc, microarray results are for a c-myc binding protein and a downstream target. ³RT-PCR results are for glucokinase regulatory protein, microarray results are for an EST similar to hexokinase domain containing 1.

liver samples on human nylon membrane arrays^[6]. Using the standard microarray protocol according to the manufacturer's instructions, we have demonstrated a large

overlap of results from the two studies as well as a high degree of similarity between the normal liver tissue from uninfected woodchucks and those of woodchucks with

HCC.

We found supporting data for 8 of the top 10 up-regulated genes and all 10 of the top 10 down-regulated genes (Table 4). The top two up-regulated genes (steroyl-CoA desaturase and elongation of long chain fatty acids, family member 6) are involved in fatty acid biosynthesis, which suggests that fatty acid synthesis is up-regulated in HCC compared with the surrounding non-cancerous liver tissues in woodchucks.

Due to the limited number of woodchuck genes that have been sequenced (fewer than 600), we could not perform RT-PCR on all of the genes of interest, and in some cases the sequence was not available for the exact gene. FACVL1, AFP, IDH3B, CPT2, and SSAT had specific probes on the microarray; ACAS2, CMYC, GCKR, and TEGT were similar to what we had microarray probes for; and there was no probe on the microarray for NMYC2. The microarray results showed expression of a hypothetical protein similar to the 5' region of ACAS2, a c-myc binding protein, a c-myc downstream target, an EST similar to hexokinase domain containing 1, and an EST similar to TEGT. Of the 66 probes on the microarrays that significantly changed in all 3 pairs of samples, we validated 2 genes with available sequence information. TEGT was up-regulated and SSAT was down-regulated in the tumors as shown by both the microarray and RT-PCR analysis. TEGT is a negative regulator of apoptosis and has been shown to be up-regulated in cancer cells^[22] and SSAT, which is the rate limiting step in polyamine catabolism, has been shown to be induced at the transcriptional level to sensitize tumors for adjuvant treatment^[23].

We also investigated genes involved in carcinogenesis and various metabolic pathways. IDH3B, the rate-limiting enzyme in the tricarboxylic acid cycle, was up-regulated in 2 probes in the same 2 tumors and expression validated with RT-PCR. A hypothetical protein similar to ACAS2, which catalyzes the conversion of acetate to acetyl-CoA for use in many metabolic and biosynthetic pathways, was up-regulated in 2 tumors according to the microarray results and shown to be up-regulated in all 3 tumors with RT-PCR. ACAS2 is of particular relevance because of the usage of the PET imaging tracer [¹¹C]-Acetate. Currently, it is not entirely known what pathways are responsible for the high uptake of acetate which can be seen in HCC compared with surrounding normal liver tissues in the PET images. We have demonstrated high up-regulation of the enzyme that catalyzes the formation of acetyl-CoA from acetate, so we hypothesize that this may be the first step involving [¹¹C]-Acetate.

An EST similar to hexokinase was up-regulated in 1 tumor according to the microarray results, and glucokinase regulatory protein (GCKR) was up-regulated in a different tumor based on RT-PCR. Sequence information for the woodchuck glucokinase gene was not available, so RT-PCR was performed on a GCKR, a negative regulator of glucokinase. Glucokinase is important for its role in PET imaging of [¹⁸F]-fluorodeoxyglucose (FDG). Glucokinase catalyzes the phosphorylation of FDG, thereby trapping it in the cell according to previous kinetic modeling data^[24]. Up-regulation of glucokinase or down-regulation of GCKR would be expected to result in higher uptake

of FDG in HCC compared with the non-cancerous liver tissues. However, our results were not conclusive and future work will require sequencing of the woodchuck hexokinase gene or examination of glucokinase regulation at the protein level.

CPT2, a protein that facilitates transport of long-chain fatty acids to the inner mitochondrial membrane for fatty acid metabolism, did not show any change according to the microarray results, but was found to be up-regulated in 2 of the 3 HCCs using RT-PCR. In the target sequence used to create the probes on the human microarray, there is a 436 residue overlap with 40.4% homology between the microarray CPT2 target sequence and the woodchuck CPT2 sequence used for RT-PCR. The lack of homology between the sequences might be the reason for not obtaining significant microarray results.

AFP, a serum marker for HCC in humans^[25], was up-regulated in one tumor and showed no change in the other two on the microarrays, while it was up-regulated in all three tumors as measured by using RT-PCR. NMYC2, which was found highly expressed in 60% of woodchuck HCCs^[26], was up-regulated in one tumor by using RT-PCR.

These results from using human Affymetrix GeneChips[®] provide a part of the foundation for our imaging work to explore the woodchuck model of virus-induced HCC. Future work will include more investigations of the regulation of pathways involving PET imaging tracers using enzyme assays and immunohistochemistry. Additional efforts will be focused on developing new radiolabeled antibodies and/or ligands for potential imaging targets found to be up-regulated in woodchuck HCC compared with surrounding non-neoplastic woodchuck hepatic tissues.

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Hepatectomy for huge hepatocellular carcinoma in 634 cases

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Abstract

AIM: To clarify the safety and feasibility of hepatectomy for huge hepatocellular carcinoma (HCC).

METHODS: A total of 4765 patients with HCC operated at Tongji Hospital were retrospectively studied, of them, 780 patients had huge HCC (10 cm or more in diameter). Hepatectomy was carried out on 634 patients (81.2%). The majority of the liver resection were major resections, and combined resection of the adjacent organs or structures was common (17.2%). The liver resection was combined with portal vein thrombectomy in 139 patients (21.9%).

RESULTS: Postoperative complications were common (26.8%) and required another laparotomy to prevent the complications in 5 patients (0.8%). The 30-d mortality was 2.2%. The main causes of postoperative deaths were liver failure ($n = 9$), postoperative bleeding ($n = 4$) and septic complication ($n = 1$). The 3-, 5- and 10-year survival rates after liver resection were 35.1%, 18.2% and 3.5%, respectively.

CONCLUSION: Hepatectomy for huge HCC is safe and effective. It should be used to treat patients with low surgical risks and resectable tumours.

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Key words: Hepatectomy; Huge hepatocellular

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide, with an annual occurrence of at least one million new cases^[1-3].

Hepatic resection is the mainstay of treatment for resectable tumours because of the proven impact of adequate tumor removal on prognosis. Recent improvements in perioperative management have made partial hepatectomy safe.

Growing indications are related to the resection extent and the approach of surgery in patients with HCC, and the maximum prognosis benefit can be combined with optimal safety. Therefore, surgeons should balance the benefits of oncological clearance against the risks of inadequate parenchymal preservation after liver resection.

A non-cirrhotic liver can tolerate up to 80% liver resection. The regenerative capacity of the liver enables a functional compensation within a few weeks and regeneration to roughly 75% of the preoperative liver volume within 1 year. Such a favourable outcome, however, cannot be extrapolated to cirrhotic liver resection which is commonly carried out for HCC.

Hepatectomy for huge HCC is associated with a higher operative morbidity and mortality because of the extent of liver resection, and a histological margin of over 1 cm is usually recommended to cut down on intrahepatic tumor recurrence^[4-6]. Of the many prognostic factors associated with long-term survival, the size of tumor has a positive association after 'curative' resection for HCC^[6-10]. The aim of this retrospective study was to find out whether liver resection for huge HCC (tumor bigger than 10 cm in diameter) is efficacious and safe.

MATERIALS AND METHODS

A retrospective study was done on 4765 patients with HCC who were treated surgically at the Tongji Hospital, Wuhan, China from January 1972 to December 2002, and 780 (16.8%) patients had huge HCC over 10 cm in diameter. Liver resection was carried out on 634 patients (81.2%).

The criteria for liver resection for huge HCC with curative intention were good general condition of the patient, good cardiopulmonary and renal functions, Pugh-Child's A and B, normal indocyanine green test, tumor confined to one lobe of the liver, and no extrahepatic spread of disease with the exception of local invasion to

Table 1 Demographic data of 634 patients with huge hepatocellular carcinoma who underwent liver resection

Sex (male:female)	563:71
Age (yr) ¹	39 ± 9.5
Total bilirubin (μmol/L) ¹	12.5 ± 2.8
Alanine aminotransferase (U/L)	36 ± 8.5
Albumin (mmol/L) ¹	3.8 ± 0.15
Prothrombin times (seconds prolonged) ¹	1.48 ± 0.8
Maximum size of tumor (cm) ¹	14.1 ± 2.6
Pugh-Child's grading, <i>n</i> (%)	
A	559 (88.2%)
B	75 (11.8%)
C	0
Alpha fetoprotein ≥ 400 ng/mL	310 (58.9%) ²
HBsAg positive	469 (74.0%)
Satellite nodules	139 (26.5%)
Tumor thrombi in portal vein	139 (21.9%)
Associated cirrhosis	547 (86.3%)

¹ mean ± SD. ² number of patient studied = 498.

adjacent organs.

Liver resection for huge liver tumours was performed as previously described^[11,12]. In brief, we routinely used the Pringle's manoeuvre to occlude the hepatic inflow. We divided the porta hepatis pedicle supplying the side of the liver to be resected as described by Launois^[13]. We then transected the liver parenchyma using finger-fracture technique with a haemostat along the plane of transection. The hepatic vein draining the part of the liver to be resected was ligated and divided intrahepatically. Then the portion of the liver to be resected by division of the ligaments was mobilized. This method of liver resection prevented laceration of the major vessels during mobilization of the liver, avoided rupturing of the huge tumor due to excessive traction, and excessive bleeding from the venous collaterals due to cirrhosis and portal hypertension by leaving mobilization of the liver near the end of liver resection. Oncological principles of ligating the vessels prior to manipulation of tumor were followed to diminish shedding of tumor cells into the blood stream.

RESULTS

Of the 780 patients with huge HCC of over 10 cm in diameter treated surgically, 634 (81.2%) underwent liver resection. The remaining 146 patients had unresectable tumors at laparotomy. The reasons for unresectability were: tumours too extensively involving the liver (*n* = 27), multiple secondary tumors (*n* = 56), severe liver cirrhosis (*n* = 44), and tumor infiltration into major vascular structures including the main portal vein, common hepatic artery or the hepatic veins (*n* = 19). These patients with unresectable HCC were treated with intraoperative transhepatic or transportal vein chemotherapy, or with chemotherapy *via* a totally implantable drug infusion system (*n* = 57), intraoperative microwave coagulation (*n* = 36), intraoperative cryotherapy (*n* = 45), or a biopsy of the liver tumor (*n* = 8).

The demographic data of the 634 patients who underwent liver resection are shown in Table 1. Majority of the patients underwent major liver resection. The terminology

Table 2 Type of operation (*n* = 634)

Type of liver resection	<i>n</i>	%
Resection of right three lobes	30	4.8
Resection of right half	44	6.9
Resection of left three lobes	39	6.2
Resection of left half	63	9.9
Segmentectomy 4, 5, 6	33	5.2
Segmentectomy 4, 5, 8	27	4.2
Segmentectomy 5, 6, 7	87	13.7
Segmentectomy 2, 3	128	20.2
Non-anatomical resections	183	28.9
Total	634	100
Combined organ resection		
Partial diaphragmectomy	12	1.9
Right adrenal gland resection	9	1.4
Distal gastrectomy	5	0.8
Transverse colectomy	11	1.7
Splenectomy	31	4.9
Splenectomy + devascularization	41	6.5
Portal vein thrombectomy	139	21.9
Total	248	39.1

of the liver resection used was included according to the recommendation by the International Hepato-Pancreato-Biliary Association. Combined resection of adjacent organs involving the tumor was common. A significant proportion of patient had tumor thrombi in the portal vein (Table 2).

The operative time was 98 ± 41 min, inflow occlusion time was 10.5 ± 2.7 min. Blood loss was 440 ± 250 mL and transfusion requirement was 480 ± 350 mL. Postoperative complications were common, such as reactive right pleural effusion (*n* = 135, 21.4%), significant ascites (*n* = 30, 4.7%), bleeding from stress ulceration (*n* = 11, 1.7%), postoperative hemorrhage on the surface of liver (*n* = 7, 1.3%), infected wound (*n* = 10, 1.6%), bile leakage (*n* = 7, 1.1%) and intraabdominal abscess (*n* = 6, 0.9%). Re-laparotomy to deal with the complications was required in 5 patients (0.8%). The 30-d mortality rate was 2.2%, 9 of 14 patients died of liver failure, 4 of 14 died of postoperative bleeding, and 1 died of septic complication. The 3-, 5- and 10-year survival rates were 35.1%, 18.2% and 3.5%, respectively (Figure 1).

Univariate analysis showed the following prognostic factors associated with significant improvement in survival: female sex, low serum alpha fetoprotein of less than 400 ng/mL, presence of tumor capsule, absence of extensive capsular infiltration by tumor, absence of tumor thrombus in main portal vein and satellite nodules (Table 3). On multivariate analysis, the significant prognostic factors for long-term survival were extensive capsular infiltration, tumor thrombus in portal vein, satellite nodules and intraoperative blood loss (Table 4).

DISCUSSION

HCC usually presents at a very late stage in its natural history because of the lack of symptoms in the early stage. The large size and the position of the liver behind the costal cartilages preclude the tumor from being readily palpable during early stage. Furthermore, the large functional reserve of the liver delays clinical presentation

Table 3 Univariate analysis on prognostic factors associated with long-term survival in patients with huge hepatocellular carcinoma undergoing liver resection

Variable	Patients (n)	Survival rates (%)				P
		1 yr	2 yr	3 yr	5 yr	
Patients						
Sex						
Male	332	65.1	43.7	33.7	15.1	P < 0.05
Female	24	83.3	70.8	45.8	37.5	
Age						NS
< 50 yr	206	64.5	42.7	32.0	15.1	
≥ 50 yr	150	68.7	46.3	38.0	18.7	
¹ AFP < 400 ng/mL	130	68.4	49.2	37.7	19.2	P < 0.05
≥ 400 ng/mL	154	62.3	32.5	26.0	9.1	
Tumor size						P < 0.05
10-15 cm	281	71.5	46.6	39.5	17.4	
> 15 cm	75	46.7	41.3	16.0	0	
Capsule						P < 0.05
Positive	270	71.0	48.1	44.0	21.9	
Negative	86	48.8	37.2	4.7	0	
Extensive capsular infiltration						P < 0.05
Positive	224	67.4	40.6	39.3	12.1	
Negative	46	93.5	84.8	76.1	69.6	
Tumor thrombus in portal vein						P < 0.05
Positive	62	38.7	22.6	6.5	0	
Negative	294	72.1	50.3	40.5	20.1	
Satellite nodules						P < 0.01
Positive	87	36.8	23.0	12.6	0	
Negative	269	75.8	52.8	41.6	21.9	
Surgical resection margin						NS
≤ 10 mm	251	64.1	40.2	34.3	13.9	
> 10 mm	105	71.4	58.1	35.2	22.9	
Intraoperative blood loss						NS
≤ 800 mL	260	69.6	43.8	33.5	15.4	
> 800 mL	96	57.3	50.0	37.5	19.8	

¹n = 498.

with functional disturbances of the liver. Some patients present with large tumors in the liver in their first visits.

The tumor size is a significant risk factor for intrahepatic and extrahepatic spread^[14-16]. Whether the tumor's greatest diameter impacts on the disease-free interval and the overall survival remains unclear. Even when small HCCs are examined as a specific group, a better survival can still be observed with decreasing size of the tumor after resection^[7,9]. The frequency of intrahepatic metastasis increases by almost a third between HCC smaller and larger than 5 cm and the rate of portal vein tumor thrombosis is almost doubled^[15,16]. There is little information showing that patients with huge HCC over 10 cm in diameter without any extrahepatic metastases have long-term results after liver resection. Our study on 634 patients showed a 5-year survival of 18.2%. It is low when compared with reported series of patients with HCC unselected for the tumor size, the overall 5-year survival of these series ranges from 11% to 76% with a median around 30%, which might be due to the more advanced stage of cancer at the time of surgery.

One of the major concerns in operating on huge HCC is the operative mortality because of the extent of the liver resection, which is commonly involved in these huge tumors. The 30-d mortality of our patients was 2.2% which is low compared with that reported in the medical literature on HCC ranging from 0.5% to 21.5%, with a median of around 5% to 10%. Our low operative mortality

Table 4 Multivariate analysis on prognostic factors associated with long-term survival in patients with huge hepatocellular carcinoma undergoing liver resection (n = 356)

Factor (variable)	Coefficient of regression	Relative risk	Standard error	Wales statistic	P
Capsule					
Positive	-1.5152	1.3521	0.5078	9.543	< 0.05
Negative					
Extensive capsular infiltration					
Positive	-2.5237	1.4152	0.4035	10.321	< 0.05
Negative					
Tumor thrombus in portal vein					
Positive	-3.1012	2.4240	0.7122	7.543	< 0.05
Negative					
Satellite nodules					
Positive	-2.5158	1.7852	0.8055	7.257	< 0.05
Negative					
Intraoperative blood loss					
≤ 800 mL	-5.5257	1.5023	0.7211	15.15	< 0.05
> 800 mL					

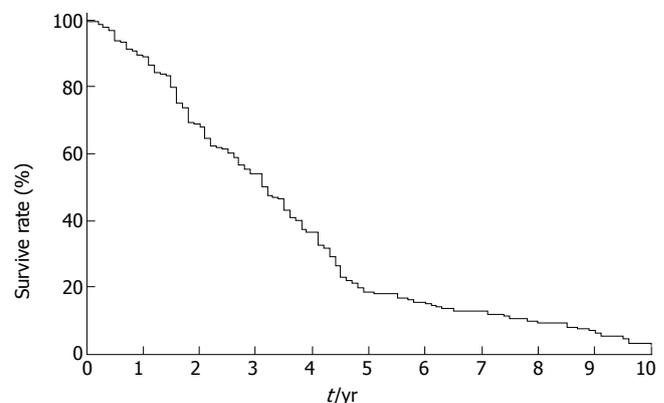


Figure 1 Survival curve for patients who underwent liver resection for huge hepatocellular carcinoma (n = 634). The 3-, 5-, and 10-yr survival rates were 35.1%, 18.2% and 3.5%, respectively.

is probably related to the surgeons' experience and careful case selection.

Intraoperative blood loss can adversely affect the perioperative mortality of patients undergoing liver resection^[18-20]. There is evidence that blood transfusion may be associated with an increased risk of recurrence in patients operated for a hepato-biliary malignancy, due to impairment of the patient's immune response^[21]. Our method of liver resection^[11,12] can offer much to patients with huge HCC. As discussed previously, our technique not only cuts down the intraoperative bleeding, but also follows oncological principles. The other factor related to our good results is careful selection of patients. In cirrhotic liver resection, the onset of postoperative liver failure and mortality is related to the degree of preoperative liver failure^[22] and the extent of non-tumor liver parenchyma removed^[23]. An ideal candidate for liver resection in a patient with huge HCC is a patient with good liver function without ascites (Pugh-Child A).

Using univariate and multivariate analyses, we identified several prognostic factors, associated with a better long-term survival for patients with huge HCC after live resection. However, all these factors reflect the biologic

behavior of the tumor, and cannot be used to exclude patients from liver resection with an intention for a cure. They can be used as a guide to make decisions in balancing the risks of the operation against the potential benefits in a patient who has increased risks of mortality because of his general condition or borderline liver functions. In cirrhotic liver resection, resecting too generous a margin can result in a higher chance of postoperative liver failure. However, a negative histological margin is the only factor that the surgeon can control the long-term survival results. Therefore the tumor should be resected aiming at a 1cm gross margin although our study showed resection margin of above or below 1 cm had no impact on long-term survival provided that the histological margin was clear.

In conclusion, in carefully selected patients, liver resection in patients with HCC of 10 cm or more in diameter is safe.

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Efficacy of ultrasonography and alpha-fetoprotein on early detection of hepatocellular carcinoma

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Abstract

AIM: To evaluate the effectiveness of ultrasonographic screening for early detection of hepatocellular carcinoma (HCC).

METHODS: The data of 14 968 patients who had ultrasonography (US) for chronic liver diseases were collected into a database program from June 1995 to June 2005. The risk factors for HCC were also studied. A total of 6089 patients who had repeated US were enrolled, 264 patients were diagnosed with HCC during follow-up (mean, 39 mo).

RESULTS: The detection rate of small HCC (≤ 3 cm in diameter) was 67.7%. The tumor size detected by screening at the intervals of 6 mo was significantly smaller than that at longer intervals. Only 29.3% of HCC patients had an elevated serum alpha fetoprotein (AFP) level above 400 ng/mL. The risk of HCC development during follow-up was higher in patients with liver cirrhosis (10.9%) and hepatitis C (9.0%) than in patients with chronic hepatitis (4.2%), hepatitis B (4.9%) and non-B, non-C hepatitis (NBNC, 3.9%).

CONCLUSION: US screening at a interval of 6 mo is beneficial to high-risk patients over 40 years old and the early detection of HCC prolongs survival.

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Key words: Hepatitis C; Hepatocarcinogenesis; Interferon; Retreatment

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INTRODUCTION

Hepatocellular carcinoma (HCC) ranks fifth in frequency of cancers worldwide^[1-3]. As the prognosis of HCC is extremely poor and effective treatment for patients with advanced HCC has not yet been established, the early detection of HCC is important for its effective treatment. Chronic hepatitis B and C as well as cirrhosis, irrespective of their etiology, are recognized as the major factors increasing the risk of HCC^[4-7]. Thus, HCC screening has been extended to include patients with chronic hepatitis B or C as those with cirrhosis^[8,9]. The most important tumor marker for HCC is alpha-fetoprotein (AFP). The common method for screening high risk patients using AFP marker can detect more early tumors and prolong the survival of patients^[10]. Although HCC screening test has become an accepted procedure among high risk populations, there are still some arguments about its effectiveness because there is no randomized controlled study showing a decrease in disease mortality^[11,12]. In addition, the usefulness, frequency and cost-effectiveness of HCC screening may differ in different areas, which may reveal a different prevalence of hepatitis B or C. The aim of this study was to evaluate the usefulness of a screening system in early diagnosis of HCC and to assess the risk factors for HCC development in patients with chronic liver diseases.

MATERIALS AND METHODS

Patients

From June 1995 to June 2005, all data of patients undergone US for HCC or chronic liver diseases in our hospital were collected into a special database program. A total of 14 968 patients had 29 926 examinations during a period of 10 years. We excluded patients with a history of liver cancer or other diseases that might affect survival. In addition, patients with focal hepatic lesion detected at admission or within 3 mo after enrollment were also excluded. All patients were periodically followed up for occurrence of any liver disease. A total of 6089 patients enrolled in this study had US periodically for at least 1 year more.

To assess the risk factors for HCC development, the clinical parameters were collected from 1982 patients. We entered all detailed data into the database program. The age distribution was mostly between the fifth and seventh decades (data not shown). Seventy-seven percent of subjects were positive for hepatitis B surface antigen (HBsAg), 15.5% were positive for anti-hepatitis C virus

Table 1 Comparison of liver status at initial screening and detection of HCC

Liver status	Patients, <i>n</i> (%)	
	At initial screening	At detection of HCC
CH	63 (23.9)	9 (3.4)
LC	201 (76.1)	255 (96.6)
Child A	85 (42.2)	122 (47.8)
Child B	38 (19.0)	58 (22.7)
Child C	78 (14.9)	75 (26.1)

Table 2 Underlying liver diseases at initial US of patients with HCC, *n* (%)

Liver status	HBV (+)	HCV (+)	BC	NBNC	Total
CH	45 (17.3)	13 (4.9)	3 (1.1)	3 (0.6)	42 (23.5)
LC	145 (54.8)	37 (14.0)	2 (0.9)	17 (6.4)	201 (76.1)
Total	190 (72.1)	50 (18.9)	5 (2.0)	19 (7.0)	264 (100.0)

Table 3 Tumor size at diagnosis

Diameter (cm)	Patients, <i>n</i> (%)
≤ 2	106 (40.8)
> 2-3	70 (26.9)
> 3-5	57 (21.9)
> 5	27 (10.4)
Total	260 (100.0)

(anti-HCV), 0.5% were positive for both and 8.4% were negative for both.

All patients were prospectively monitored by measurement of serum AFP and US at the intervals of 3 mo or 12 mo according to the status of underlying liver disease. The mean follow-up time was 39 ± 28 mo. The duration of HCC development was calculated by the time interval between the date of initial US and diagnosis of HCC. When US showed a new focal lesion or the serum AFP was increased, additional investigations were performed, such as a repeated AFP test, contrast computed tomography, or magnetic resonance imaging. HCC was diagnosed by histology or typical imaging features and elevated serum AFP level (≥ 400 ng/mL).

Serum AFP level was measured by a commercial enzyme-linked immunosorbent assay kit (Abbott, North Chicago IL, USA). US was performed by internal physicians.

Statistical analysis

The data were analyzed using the chi square test, logistic regression, or Kaplan-Meier method. All statistical analyses were done with SAS program and SPSS for windows (7.5.1) (Kaplan-Meier method and Cox regression hazard model).

RESULTS

HCC was found in 264 patients by screening during follow-up, and the annual detection rate was 2.1%. At

Table 4 Tumor size according to US interval

US interval (mo)	Patients, <i>n</i> (%)	Tumor size (cm)
≤ 6	124 (47.2)	2.7 ± 1.3^a
≤ 3	26 (10.0)	3.4 ± 1.8
4-6	98 (37.2)	2.5 ± 1.1
> 6	140 (52.8)	3.4 ± 2.0
7-12	90 (34.0)	3.0 ± 2.1
> 12	50 (18.8)	4.4 ± 1.7
Total	264 (100.0)	3.1 ± 1.7

^a*P* < 0.01 vs > 6-mo group.

Table 5 Median survival according to tumor size

Diameter	Patients, <i>n</i> (%)	Median survival (mo)
≤ 2	106 (40.8)	42
> 2-3	70 (26.9)	35
> 3-5	57 (21.9)	20
> 5	26 (10.4)	10
Total	260 (100.0)	31

Table 6 Serum AFP level at detection of HCC

AFP level (ng/mL)	Patients, <i>n</i> (%)
≤ 20	107 (41.1)
≥ 1-400	86 (29.6)
> 400	70 (29.3)

enrollment, cirrhosis was found in 201 patients (76.1%), chronic hepatitis was diagnosed in 63 patients (23.9%). However, most patients (96.6%) progressed to liver cirrhosis when HCC was detected (Table 1). Of the 264 HCC patients, 190 (72.1%) were associated with hepatitis B, 50 (18.9%) with hepatitis C, and 19 (7.0%) not associated hepatitis B or C (Table 2).

The mean diameter of tumor at diagnosis was 3.1 ± 1.7 cm, and the detection rate of small HCC (≤ 3 cm) was 67.7%. Of the 260 patients, 27 (10.4%) had a tumor larger than 5 cm in diameter (Table 3). The mean diameter of the detected tumors at the intervals of 6 mo was significantly smaller than that at longer intervals (2.7 cm vs 3.4 cm, *P* < 0.01). However, there was no difference in tumor size at different time intervals (Table 4). The median survival time was 31 mo (Table 5) and the smaller the tumor size the longer the survival time. Of the 263 HCC patients, 70 (29.3%) had an elevated serum AFP level above 400 ng/mL (Table 6). However, the serum AFP levels were below 20 ng/mL in 107 patients (41.1%).

The incidence of HCC was higher in patients with liver cirrhosis (10.9%) than in those with chronic hepatitis (4.2%) and HBsAg carriers (1.5%). No patient with fatty liver developed HCC. The incidence of HCC was higher in hepatitis C-related group (9.0%) than in hepatitis B- (4.9%) and NBNC-related group (3.9%). However, there was no significant difference between hepatitis C and B patients older than 40 years.

DISCUSSION

The prognosis of HCC is poor especially in Africa and China^[13]. By contrast, the disease runs a more benign course in patients in low risk regions, although they have a mean survival time of only about 6 mo^[14]. The role of chronic infection with HBV and HCV in the etiology of liver cancer is well established^[15]. Some 360 million people are chronically infected and at risk of death due to cirrhosis and HCC. Five hundred thousand to 700 000 people die of HBV-related liver diseases each year^[16]. More than 170 million people worldwide are chronically infected with HCV, which is responsible for more than 100 000 cases of liver cancer per year^[17].

The usefulness, frequency, and cost-effectiveness of HCC screening may differ in different geographic areas or in different underlying liver disease populations because there may be differences in the incidence and growth characteristics^[9,11]. Although US screening for HCC is still controversial, it is a generally accepted strategy in East Asian countries. As patients with chronic hepatitis or cirrhosis may develop HCC, emphasis has been placed on the early detection of HCC when it is small, asymptomatic and potentially curable, by screening patients at high risk^[18]. Several trials in areas of high and low HCC incidence have demonstrated that screening can detect patients at early stage of the disease, increase the resection rate and prolong survival time. Randomized prospective studies in China on high risk patients showed that the survival rate of patients after HCC resection is 52.7% after three years^[19,20].

Cirrhosis is recognized as the major risk factor for HCC, and the annual risk of developing HCC is between 1% and 6%^[21-25]. In our study, the annual risk of HCC development in cirrhosis patients was 3.5%. Reported screening studies showed that 20%-56% of HCC patients have undiagnosed cirrhosis^[26,27]. Screening has been extended to include patients with chronic viral hepatitis as well as those with cirrhosis. The overall annual detection rate of HCC in surveillance studies including chronic hepatitis varies from 0.8% to 4.1%^[11,28-31]. In our study, the annual detection rate of chronic hepatitis was 1.19%.

If the sample size of the target population or the number of HCC cases is not large enough, it is difficult to evaluate the effectiveness of screening in a high-risk population with different status and etiology of liver diseases. Therefore, a large target population and a sufficient number of HCC cases can avoid sampling bias. In our study, the study subjects were enrolled from 29 926 patients who had US for HCC screening or for various chronic liver diseases. We enrolled 6089 patients who had US periodically for a period of at least 1 year by using a database program. Our study included a variety of liver diseases such as hepatitis B or C, fatty liver and liver cirrhosis-related hepatitis B and C, or non-B and C. Two hundred and sixty-four patients were found to have HCC during a mean 39-mo follow-up, and the annual detection rate was 2.0%.

As the sample size was large enough to allow estimation, we tried to evaluate the efficacy of HCC screening. Because our study was a clinic-based screening,

the incidence of HCC was higher than that of Sherman *et al*^[9]. In our study, 76.9% of patients with HCC had liver cirrhosis and 23.1% had chronic hepatitis. Furthermore, most of these cases progressed to liver cirrhosis before development of HCC. Although the incidence of HCC was higher in liver cirrhosis patients (10.7%) than in chronic hepatitis patients (4.0%), chronic hepatitis B or C might progress to cirrhosis and HCC, suggesting that surveillance is needed to include patients with chronic viral hepatitis over the age of 40 years in China.

Reported screening intervals vary from 3 to 12 mo. A 6-mo interval is generally accepted as a rational choice. However, some others prefer shorter intervals in high-risk patients^[32,33]. In our study, the mean diameter of tumor size at diagnosis was 3.1 ± 1.7 cm and the detection rate of small HCC (< 3 cm) was 67.7%. However, there was no difference in tumor size between different time intervals (Table 4). Although a shorter screening interval can detect earlier HCC, it decreases cost-effectiveness.

Whether early detection of tumors can prolong survival time remains unclear^[11]. In our study, the overall median survival of the screened group was 31 mo, suggesting that survival time correlates well with tumor size. Furthermore, survival time was remarkably improved when the detected tumor size was equal or smaller than 2 cm (Table 5). Although the resection rate was relatively low in our study, most tumors, especially those less than 3 cm in diameter, were amenable to non-surgical curative or effective therapy.

Only 70 of the 264 HCC patients (29.3%) had an elevated serum AFP level above 400 ng/mL. In addition, the serum AFP level was below 20 ng/mL in 41.1% of patients. Trials in China have shown that combination of US and AFP can achieve better results in screening HBsAg positive subjects^[21,34].

The incidence of HCC was higher in liver cirrhosis patients (10.9%) than in chronic hepatitis patients (4.2%). None of the patients with fatty liver developed HCC. The incidence of HCC was higher in hepatitis C-related group (9.0%) than in hepatitis B- (4.9%) and NBNC-related groups (3.9%). However, the incidence was not significantly different between hepatitis C and B in the group over 40 years of age. As the risk of HCC is negligible in hepatitis B patients under the age of 30 years and in hepatitis C patients under the age of 40 years, the screening would be better restricted to patients over the age of 40 years.

In conclusion, US screening at the intervals of 6 mo is beneficial to high-risk patients over the age of 40 years and prolongs survival. According to risk factors, the necessity for a screening test and a proper interval should be reconsidered.

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COLORECTAL CANCER

Expression of NOS and HIF-1 α in human colorectal carcinoma and implication in tumor angiogenesis

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Abstract

AIM: To study CD34, CD105, inducible nitric oxide synthase (iNOS), endogenous nitric oxide synthase (eNOS), and hypoxia-inducible factor 1 (HIF-1) α expression in human colorectal carcinomas.

METHODS: The tissue microarrays (TMAs) were made up of 80 cases of colorectal carcinoma and 80 cases of non-neoplasm colorectal mucosa. The expression of CD34, CD105, NOS and HIF-1 α was detected by immunohistochemistry (S-P).

RESULTS: iNOS and HIF-1 α expression in colorectal carcinoma was significantly higher than in non-neoplasm colorectal mucosa ($\chi^2 = 43.166, P < 0.01$; $\chi^2 = 10.4278, P < 0.01$); eNOS expression in colorectal carcinoma was significantly lower than in non-neoplasm colorectal mucosa ($\chi^2 = 11.354, P < 0.01$). The expression of iNOS correlated with differentiation ($\chi^2 = 18.141, P < 0.01$), invasive depth ($\chi^2 = 4.748, P < 0.01$), and Micro vessel density (MVD) ($t = 2.327, P < 0.05$). The expression of HIF-1 α was correlated with infiltrating depth ($\chi^2 = 4.397, P < 0.05$), Duke's staging ($\chi^2 = 4.255, P < 0.05$), and MVD ($t = 2.272, P < 0.05$). No correlation was found in eNOS expression.

CONCLUSION: Over-expression of iNOS and HIF-1 α in colorectal carcinoma is correlated with the biological character MVD.

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Key words: Nitric oxide synthase; HIF-1 α ; Colorectal carcinoma; Angiogenesis

INTRODUCTION

Angiogenesis is an important step in the growth and metastasis of solid malignant tumors. To adapt to hypoxia, angiogenesis is essential for tumor growth. Tumor must over-express genes that encode angiogenic factors to induce new blood vessels^[1]. A number of angiogenic factors have been found, and there is interrelation among them, which has been widely researched. Nitric oxide synthase (NOS) and hypoxia-inducible factor 1 (HIF-1) α are two of these factors which may be correlated with angiogenesis. To screen out angiogenic factors is time-consuming and expensive. Tissue microarray (TMA) brings to pathologists a completely new method to solve this problem. TMA has many excellent features such as small volume, high information content, and assembling differently according to different needs. A number of results can be obtained through one experiment^[2], which reduces research time and improves efficiency greatly. Moreover, the most important advantage is a decrease of experimental error, because TMA can make all samples under the same experimental condition. To study NOS and HIF-1 α as angiogenic factors, we used immunohistochemistry to detect the expression of CD34 and CD105 and the expression of inducible NOS (iNOS), endogenous NOS (eNOS), and HIF-1 α in human colorectal carcinomas. TMAs consisting of 80 colorectal carcinoma and 80 non-neoplasm colorectal mucosa were employed to accomplish these studies.

MATERIALS AND METHODS

Patients

Eighty specimens were collected from 80 patients with colorectal carcinoma (median age 62.2 ± 5.9 years, range 29-88 years) who were operated in Qingdao Municipal Hospital (Shandong, China) from Oct 2003 to Mar 2004. Carcinoma and non-neoplasm colorectal mucosa (located at least 8 cm away from the margins of cancers) were taken from each sample. There were 6 cases of well-differ-

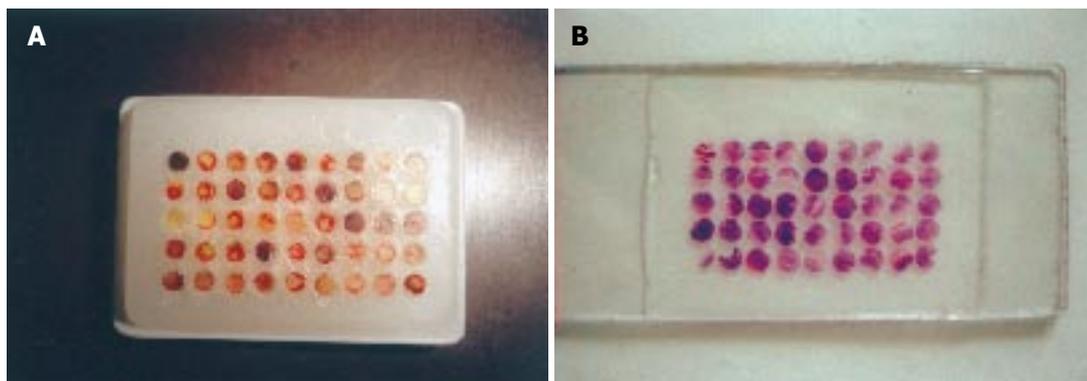


Figure 1 Tissue microarray. A: Block; B: Section (HE).

entiated adenocarcinoma, 59 cases of moderately differentiated adenocarcinoma and 15 cases of poorly differentiated adenocarcinoma. Among them, 30 cases had regional lymph node metastasis and 12 cases had distant metastasis. None of the patients received any chemotherapy or radiation therapy before surgical operation. The pathological diagnosis was made on the basis of the size, infiltrating depth, histological grade of the carcinoma, and lymph node and distant metastasis. The grading standard was in accordance with that established in the book *Practical and Surgical Pathology*^[5].

Tissue microarray

HE staining sections of all samples were observed firstly, and typical lesion positions were marked. Then the corresponding positions to these lesions were marked on paraffin wax. The empty TMA paraffin wax (45 mm \times 28 mm \times 7 mm) was holed through Tissue Arrayer (each diameter was 2 mm), and the tissues taken from marked positions were put in these holes tidily (each diameter was also 2 mm). The process was repeated for many times and the TMA paraffin wax was ready (Figure 1A). A different kind of tissue was put in it as a marker, which was kidney tissue in this experiment. Each TMA paraffin wax was sliced into 4 μ m thick sections.

Immunohistochemistry

The expression of CD34, CD105, NOS and HIF-1 α was stained by immunohistochemistry (S-P). The reagent kits were purchased from Fuzhou Maxim Co. Micro vessel density (MVD) was counted with different vessel markers CD34 and CD105 respectively. CD34 and CD105 antibodies were supplied from Neomarker Company, and working consistence was 0.2 mg/L, and 0.4 mg/L. iNOS and eNOS multi-clonal antibody was purchased from Neomarker Company; working consistence was 20 mg/L, and 20 mg/L, respectively; multi-clonal HIF-1 α antibody was from Santa Cruz Company; working consistence was 0.8 mg/L. Homogenous animal IgG that contained the same protein quantity was used instead of first antibody in negative control.

NOS and HIF-1 α staining was classified into two groups. Positive group includes: +++, most carcinoma cells were stained with a very strong intensity, and distributed in clusters; ++, a large number of carcinoma cells were stained with a moderate intensity, and distributed in

clusters occasionally; +, a few carcinoma cells were stained with a slight intensity. Negative group is defined as: -, no staining of carcinoma cells.

Counting of MVD in carcinoma tissues was in accordance with Weidner's standards with a minor modification. The sections were searched for the hot spots rich in vessels, which were located in or near the area of tumor tissues under a low power microscope (100 \times). MVD was counted under a high power (400 \times) microscope according to the standards that any stained endothelial cell or cells were identified as an independent vessel. These vessels must be clearly separated from each other. However, apparent vasa or vasa with red blood cells could be regarded as vessels. Three different HP vision fields were chosen on each of the sections. Stained vessels were counted by two pathologists under multi-ocular microscope simultaneously. The results were averaged as the relative value of the amount of vessels per unit area.

Statistical analysis

The difference of MVD between CD105 and CD34 staining was analyzed with paired sample *t*-test. The relation between MVD and expression of NOS and HIF-1 α was analyzed with *t*-test. Pearson-chi-square test was used for statistical analysis of the relation between clinical-pathological parameters and expression of NOS and HIF-1 α .

RESULTS

Tissue microarray evaluation

Every TMA section consisted of 36-46 samples, which were arrayed orderly. The shape of these samples was rounded or elliptoid, and a few samples were crimped or lost (Figure 1B). After TMA wax was sliced consecutively, one section was picked out for HE staining to make sure that the tissue still kept its histological feature. In fact, almost all samples could keep their histological feature in TMA wax which total depth was about 3 mm. Adjacent sections that contained all samples were selected. After antigen retrieval and IHC staining, 1-2 samples fell off from the sections. Therefore, the number of available samples was 78, and the availability was 97.5%.

Expression of CD34/CD105 and MVD

CD105 was mainly expressed in cytoplasm and plasmalemma of newborn endothelial cells. These positive cells were

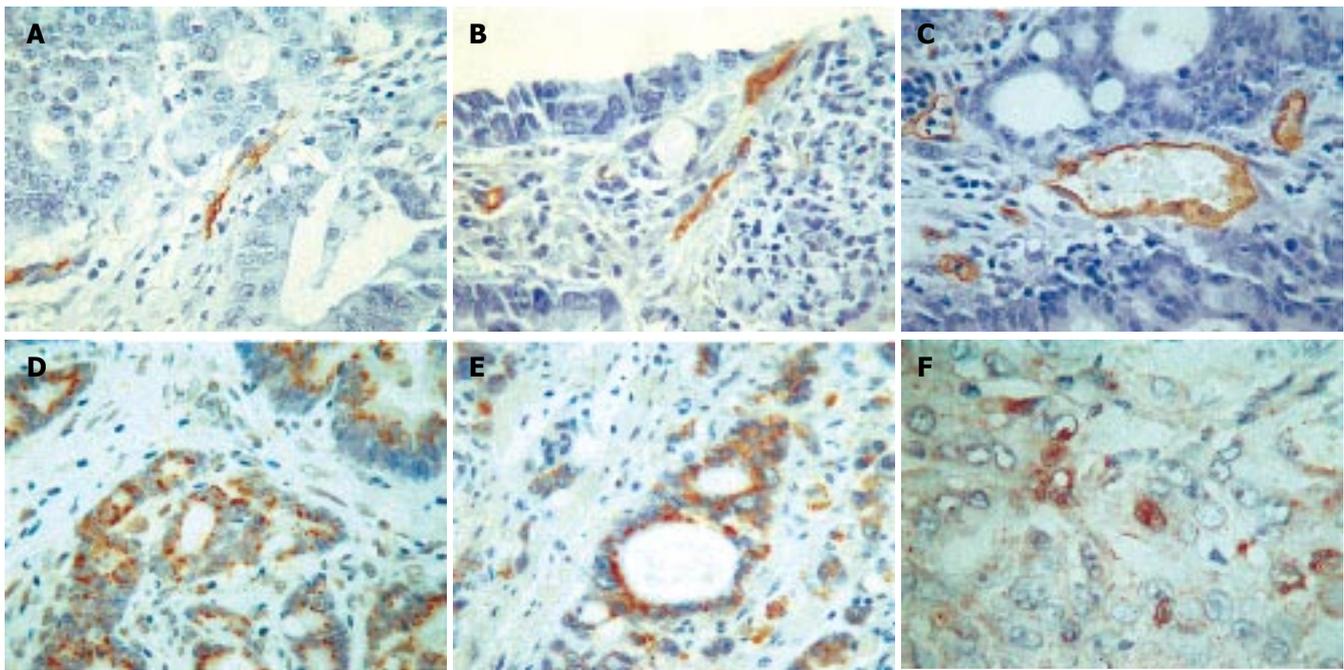


Figure 2 CD105, CD34, iNOS, eNOS and HIF-1 α expressions in colorectal carcinoma (SP \times 400). **A:** CD105 in vessels; **B:** CD105 not in vessels; **C:** CD34; **D:** iNOS; **E:** eNOS; **F:** HIF-1 α .

shown as one cell or a group of cells, and most of them ranged in a line or an irregular goblet while a few of them formed lumen. CD105 was weakly expressed or absent in native blood vessels with thick walls or large lumina (Figure 2A). The vessels with positive CD34 staining formed lines or had obvious lumina. Endothelial cells of many large vessels with thick walls and smooth muscles showed positive staining, which was distinct from the sections of CD105 staining (Figure 2B and C). *t*-test showed that MVD by CD34 staining was significantly different from that by CD105 staining (27.5 ± 10.3 vs 14.0 ± 5.8 , $t = 4.782$, $P < 0.01$).

Expression of iNOS and eNOS

The positive rate of iNOS was 78.2% (61/78) in colorectal carcinoma, and 25.6% (20/78) in non-neoplasm mucosa of colorectal tissue. The difference between them was significant ($\chi^2 = 43.166$, $P < 0.01$). The positive granules of iNOS were mainly located in cytoplasm diffusely (Figure 2D). The positive rate of iNOS decreased with the descending of differentiation grade. Most ring carcinoma showed negative expression. The expression of eNOS showed positive or weak positive in non-neoplasm mucosa of colorectal tissue. The positive rate was 60.3% (47/78). The positive granules also located diffusely in cytoplasm, and some expressed in cell nucleus (Figure 2E). The expression of eNOS in colorectal carcinoma was 33.3% (26/78), which was statistically different from that in non-neoplasm mucosa of colorectal tissue ($\chi^2 = 11.354$, $P < 0.01$). The positive rate of eNOS also decreased with the descending of differentiation grade. (Table 1)

Expression of HIF-1 α

The positive rate of HIF-1 α was 69.2% (54/78) in colorectal carcinoma, and 43.6% (34/78) in non-neoplasm mucosa

Table 1 Relation between expression of iNOS, eNOS, HIF-1 α and clinical pathology, *n* (%)

Pathology	<i>n</i>	iNOS	eNOS	HIF-1 α
Histological grade				
Well differentiation	6	5 (83.3) ^b	3 (50)	2 (33.3)
Moderate differentiation	58	51 (87.9)	21 (36.2)	43 (74.1)
Poor differentiation	14	5 (35.7)	2 (14.3)	9 (64.3)
Infiltrating depth				
Serous membrane (-)	9	4 (44.4) ^d	2 (22.2)	3 (33.3) ^a
Serous membrane (+)	69	57 (82.6)	24 (34.8)	51 (73.9)
Lymph node metastasis				
(+)	29	24 (82.8)	11 (37.9)	23 (79.3)
(-)	49	36 (77.9)	15 (33.3)	31 (63.3)
Distant metastasis				
(+)	12	11 (91.7)	5 (41.7)	11 (91.7)
(-)	66	50 (75.8)	21 (31.8)	43 (65.2)
Duke's stage				
A, B	45	35 (77.8)	18 (40.0)	27 (60) ^c
C, D	33	26 (78.8)	8 (24.2)	27 (81.8)

^a: $P < 0.05$ between serous membrane positive group and negative group;

^c: $P < 0.05$ between Duke's stage A&B group and C&D group;

^b: $P < 0.01$ between different histological grade groups;

^d: $P < 0.01$ between serous membrane positive group and negative group.

of colorectal tissue ($\chi^2 = 10.4278$, $P < 0.01$). The positive granules mainly located in cell nucleus and cytoplasm (Figure 2F). A small number of HIF-1 α granules were expressed in inflammatory cells in the stroma of tumor.

Relation between NOS/HIF-1 α and MVD

Pearson-Chi-square test showed that the expression of iNOS was correlated with differentiation ($\chi^2 = 18.141$, $P < 0.01$), and infiltrating depth ($\chi^2 = 4.748$, $P < 0.01$). The

expression of eNOS had no significant difference in different groups. However, the expression of HIF-1 α was correlated with infiltrating depth ($\chi^2 = 4.397$, $P < 0.05$), and Duke's staging ($\chi^2 = 4.255$, $P < 0.05$). MVD of iNOS positive group was significantly higher than that of the negative group (14.3 ± 6.1 vs 10.6 ± 4.0 , $t = 2.327$, $P < 0.05$); HIF-1 α staining showed the same result (15.2 ± 5.4 vs 11.5 ± 4.3 , $t = 2.272$, $P < 0.05$), while the MVD between the two groups of eNOS staining had no significant difference (13.8 ± 5.4 vs 11.9 ± 4.7 , $t = 1.523$, $P > 0.05$).

DISCUSSION

Angiogenesis refers to the formation of new blood vessels from native blood vessels, which is very important to the growth and invasion of malignant solid tumors^[4]. In 1971, Folkman firstly put forward the viewpoint that the growth of tumor depends on angiogenesis^[5]. In tumor microenvironment angiogenesis needs many steps, which is a continuous process. The characteristics of micro vessels in tumors are different from that developed under normal physiological conditions. In fact, MVD has been extensively investigated and was found to be a useful prognostic marker in many types of cancers^[6].

At present, most researchers use immunohistochemistry (IHC) to study new blood vessel in tumors. Therefore it is necessary to select an appropriate marker of endothelial cells^[7]. Previous reports have demonstrated that MVD-CD34 is a better prognostic marker than MVD assessed by many other endothelial markers, such as von Willebrand factor (vWF), CD31. However, CD34 is a pan-endothelial cell marker, thus it is difficult to distinct newborn vessels from native vessels, and it may not be able to reflect the exact angiogenesis activity in a tumor. According to some previous reports, CD105 is an endothelial marker that appears to react only with the endothelial cells in the newly formed vessels, and in particular, the immature tumor blood vessels^[8]. Based on our study, we suggest that CD105 has a higher rate of positivity and stronger reactivity on the endothelial cells of new blood vessels^[9]. Therefore CD105 is a better marker for the endothelial cells of new blood vessels. In addition, CD105 monoclonal antibody in the experiment was suitable for paraffin sections. It overcame the limit that usual antibody is only used in frozen sections. Because frozen sections were more difficult to observe than paraffin sections, it led to the inconvenience for MVD counting under microscope. Using this antibody, we saved time and materials, and obtained better staining effect. Moreover, the result of the experiment was more reliable and accurate.

NOS includes eNOS and iNOS, which have different functions. eNOS is involved in many physiological functions of neural system, such as regulating the angioectasis and smooth muscle hyperplasia. iNOS expression in human diseases has long been investigated. NO production by iNOS in tumor infiltrating macrophages may be part of their anti-tumoral cytotoxic potential^[10]. On the other hand, the expression of iNOS in endothelial cells of tumor vessels and in cancer cells itself supports the assumption that the cancer uses this form to regulate the tumor vascularisation and blood flow.

eNOS exists in normal condition and only produce a slight number of NO, while iNOS is induced by activated cells to produce more NO which can promote the construction of tumor vessels. NO has been found to be a mediator of angiogenesis and blood flow^[11,12].

Hypoxia-inducible factor 1 (HIF-1) is a heterodimeric transcriptional factor composed of two basic-helix-loop-helix (bHLH)-PAS α and β subunits. HIF-1 α is the unique, O₂-regulated subunit that determines HIF-1 activity^[13]. A series of genes and proteins that may increase the survival of tumor cells under hypoxia conditions, including vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF), iNOS, platelet-derived endothelial growth factor (PDEGF), glucose transporter 1, lactate dehydrogenase, erythropoietin and nitric oxide synthase gene, are regulated by HIF-1 α . Thus, HIF-1 α may play important roles in tumor progression. Furthermore, HIF-1 α is a critical factor of transcription in the pathway of vascular growth signals. It can activate many genes of angiogenic factors, which can lead to angiogenesis. Most studies about the effects of HIF-1 α on the neovascularization in malignancies have been done *in vitro* or in animal models. It has been confirmed that in many cancer cell lines, hypoxia can induce the expression of some proangiogenic factors, including VEGF, IGF, and PDEGF, through HIF-1 α -dependent manner. It has also been proven through the method of gene knockout that the loss of HIF-1 α may significantly suppress the growth of tumors including glioblastoma and malignant teratoma and most importantly, it may decrease the neovascularization of those malignancies. This provides a new approach for the treatment of tumor^[14].

Tissue chip is also called tissue microarray, which is a special biological chip invented by Kononen in 1998^[15]. Its principle is to remove hundreds of minute tissues from different primary blocks and place them into one arrayed block with high density. Tissue microarray is suitable for all analyses *in situ* such as antibody staining, immunohistochemistry, fluorescence *in situ* hybridization, Western blot. Thus we can investigate the different expression of tissues either at DNA and RNA level or protein level. Tissue microarray is characterized with small bulk and high-throughput, which can produce many results through one experiment^[2,16]. It can decrease experiment errors, greatly improve the efficiency of histopathological study, save experimental materials and reagents, and at the same time make the results more reliable and comparable^[17-19]. Bubendorf *et al.*^[20] made a large amount of tissue microarrays of tumor, in which ten blocks had 4788 samples from 130 kinds of tumor. These microarrays can be used to analyse the difference of newly found genes between normal tissues and tumor, from which we can acquire reliable results. By using the same method, Fernebro *et al.*^[21] also found that Ki67 positive cell index of rectal carcinoma was 0.5-1.0 (average 0.85) by the method of microarray, being not significantly different from that of routine examination method (0.54-1.0, average 0.81); moreover, both methods had the same result on p53 (average 75%). These indicate that we could get reliable and appropriate results with tissue microarray for IHC staining. We have made tissue microarrays consisting of 80 cases of colorectal carcinoma, each of which has 40-50

samples, arranging orderly. Its shape is round or oval, with few crimples and it does not fall off easily. By using tissue microarrays, it is feasible to examine clinical tissue samples on large-scale and with high efficiency, which is rapid, convenient, economical and precise.

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Mental and physical symptoms associated with lower social support for patients with hepatitis C

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Abstract

AIM: To systematically examine the impact of the hepatitis C virus (HCV) diagnosis on patients' level of social support in a large-scale study.

METHODS: Patients evaluated and treated for HCV in a tertiary referral center were enrolled in a cross-sectional study. Demographic data, functional and emotional status as measured by the Hospital Anxiety and Depression Scale (HAD) and the Sickness Impact Profile (SIP), severity of liver disease, mode of acquisition, and physical and psychiatric comorbidities were collected from patients or abstracted from the medical record. All participants completed a semi-structured interview, addressing questions of social support.

RESULTS: A total of 342 patients (mean age 45.2 years; 37% women) were enrolled. Ninety-two (27%) patients described lower levels of support by family and friends. Nearly half of the participants (45%) noted the loss of at least one relationship due to the disease. Fears related to transmitting the disease (25%) were common and often associated with ignorance or even discrimination

by others (19%). Nearly one fifth of the patients did not share information about their disease with others to avoid being stigmatized. Lower levels of social support were significantly associated with living alone, being unemployed, being excluded from antiviral therapy, having psychiatric comorbidities, contracting HCV through intravenous drug use, having high levels of anxiety and depression as measured by the HAD and negative mood state as measured by the SIP. Patients reporting lower levels of social support also noted more physical symptoms as measured by the SIP.

CONCLUSION: Patients with hepatitis C often face significant social problems, ranging from social isolation to familial stress. The most common concerns reflect a limited insight of patients and their relatives and friends about the disease, the risk factors for its spread, and about potential consequences. Our data suggest that educational interventions targeting support persons and the stressors identified in our findings may lessen or alleviate the social strains patients with hepatitis C experience.

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Key words: Hepatitis C; Social support; Depression; Anxiety; Quality of life; Stigmatization

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INTRODUCTION

Hepatitis C virus (HCV) infection, a common and slowly progressing liver disease, negatively affects quality of life. Most studies addressing the important impact of HCV focus on patients undergoing antiviral therapy^[1-6]. However, even HCV-infected individuals outside of treatment have lower quality of life scores compared to uninfected controls^[7-9]. Negative social interactions may play a unique role as a determinant of quality of life in patients with HCV, as they often face stigmatization in their professional and private environment^[10-12]. Patients living with AIDS and stigmatizing diseases such as tuberculosis and leprosy

experience a deterioration or even complete collapse of their social support network^[13-15]. Despite recent evidence of negative stereotyping in patients with HCV, few studies have focused on the effect of the disease on the patients' interactions with family, friends or other important groups^[16]. Relatively little is known about possible correlations between social support and demographic and psychosocial or physical characteristics, or of the specific difficulties that patients experience in their lives^[17]. In this analysis we hypothesize that poor social support is associated with emotional and physical difficulties in patients with HCV both in and outside of treatment. Using mixed quantitative and qualitative methods, we propose to characterize the nature and the causes of social problems as an important first step in alerting healthcare providers of the major stressors these patients face.

MATERIALS AND METHODS

Patients

The schedules of all hepatologists practicing at a large Midwestern teaching hospital were reviewed daily between October 1998 and May 2003 for patients meeting the study inclusion criteria. Patients younger than 18 years of age, prisoners, those unable to communicate verbally, or provide informed consent were excluded from the study. No exclusions for concurrent liver disease (hepatitis A or B) or HIV status were made. Patients with a confirmed diagnosis of HCV were invited to participate on the day of their clinic visit.

Data collection

Once informed consent was obtained, participants were asked to provide demographic information, to undergo an extensive interview addressing psychosocial and health-orientated questions (see Appendix), and to complete health status and psychosocial survey measures. The interview was designed to range in length from 30 min to two hours. Each interview was recorded with a hand-held tape recorder and transcribed verbatim. After the interview, patients provided demographic information (age, gender, race, marital status, education, population of home town), and completed the Hospital Anxiety and Depression Scale (HAD), a self-assessment scale for mood disturbances in a non-psychiatric patient population^[18], and the Sickness Impact Profile (SIP), a behaviorally based health status measure including sub-scales for physical symptoms (Body Care and Movement, Ambulation, Mobility), psychosocial difficulties (Emotional Balance, Social Interaction, Alertness Behavior, physical ability to Communicate), as well as physical and psychosocial summary scores^[19].

A physician abstracted from the electronic medical record patients' psychiatric diagnoses, Charlson Comorbidity Index^[20], Child-Pugh score, dates of treatment for HCV infection (before, during, after interview), substance abuse, mode of transmission (drug use, blood transfusion/needle stick, tattoo/other/unknown), and response to treatment (durable responder, nonresponder/disease recurrence, naïve). Patients who were naïve either self-excluded from treatment or were

excluded based on physical comorbidities, psychiatric disease, or substance use. Based on clinical records from the attending hepatologist, no patient was determined to remain naïve based on a lack of social support.

Coding

For the construction of our codebook^[10,21] we used the quasi-statistical method as outlined by Miller and Crabtree^[22]. We also employed the qualitative computer software program Atlas.ti (version 4.2, Berlin, Germany) to aid in the management and analysis of data. Twenty trained interviewers, each with over one year of experience in interviewing, read 50 out of 150 randomly selected interviews and ranked the top ten themes in terms of overall frequency and importance. The primary investigator and two trained coders then synthesized this list to create the master codebook which included the central themes related to social support, stigmatization, and quality of life. Using this codebook, each transcript was then coded by two independent coders, one who had undergone a minimum of three weeks formal training and a master coder with at least one-year experience. The initial coding sheets of each coder were converted into a spreadsheet to determine a final kappa score that assessed the agreement between the evaluators. The coders then met and processed the codes for each case until 100% agreement was achieved between them. From this a master code databank was developed which was then used for the statistical calculations.

For this analysis, the chief code was for social support, noting where patients described a dominantly negative environment (coded as 'unsupportive') versus all others (coded as 'supportive'). We defined social support as involving sexual partners, family members, and friends. An 'unsupportive' social environment was defined as dominantly negative (e.g., 'They have basically excluded me; so I'm all alone now,' 'Relations with my husband are just not good any more.'). Conversely, a 'supportive' social environment was defined as either positive or mixed (e.g., 'They are always there for me,' 'My in-laws won't have me in the house, but my wife and kids are okay with it.'). When the interview was too conflicted or when too few details were provided, the code of 'cannot judge social situation' was used. These interviews were then excluded from the final analysis. The ordinal code of social support had an inter-coder reliability kappa score of 0.72, or what Landis and Kock label as a 'substantial' agreement between coders^[23].

Two coders then conducted a close textual analysis of each discussion of social support present in the interviews. For the purpose of this analysis we concentrated on two main domains: (1) the negative impact of HCV on social support, and (2) the reasons for this negative impact.

Statistical analysis

All analyses were performed using SPSS version 12.0 (SPSS, Inc. Chicago, Illinois, USA). We first summarized the demographics and clinical characteristics of the patient sample, excluding anyone with missing data on social support. We then examined demographic, psychosocial,

and clinical characteristics of the patient sample, both overall and by level of social support, treating the level of social support as binary ('supportive' versus 'unsupportive'). We tested for differences between groups using ANOVA for the means of continuous variables (age, Child-Pugh score, HAD, SIP) and Chi-Square and Fisher's Exact for categorical variables, where appropriate. We then examined factors associated with social support. $P < 0.05$ was considered statistically significant.

RESULTS

Patient characteristics

A total of 499 patients were approached, with 96 declining to participate, largely due to time constraints. Of the 403 patients successfully entered into the study, 61 were excluded due to either a technological failure in recording the interview ($n = 12$) or an inability to judge the social support code ($n = 49$). This left 342 patients in the total sample with a code for social support. This sample was (Table 1) predominantly male, white, and had acquired the disease mostly through intravenous drug use. Two hundred and fifty patients (73%) were categorized as having a 'supportive' and 92 (27%) as having an 'unsupportive' environment. Of all the patients in the study only 4 (3 with hepatitis B and 1 with HIV) had another chronic, sexually transmittable disease.

Bivariate analysis

Considering the cross-sectional design, participants were interviewed at different points during the evaluation of and/or treatment for HCV (pretreatment, treatment, post-treatment, naïve). Naïve patients (typically excluded from treatment due to psychiatric illness or substance use) reported more negative social experiences compared to patients in the pretreatment, treatment, and post-treatment groups (Table 2). Marital status (living without partner) and employment status (unemployed) were significantly associated with level of social support, while all other demographic and disease-related variables did not correlate. Psychiatric comorbidity and prior substance abuse as the mode of acquisition for HCV were associated with lower levels of support. The category of unsupportive social support also correlated with greater physical symptoms as measured by the physical summary scores of the SIP and all physical sub-scales (Body Care and Movement, Mobility, Ambulation). Similarly, lower support was associated with the psychosocial summary scores and all psychosocial sub-scales of the SIP (Emotional Balance, Social Interaction, Alertness, Communication), as well as higher scores for the depression and anxiety scales of the HAD (Table 2).

Thematic analysis

Two domains emerged as patients discussed their problems. First, they described the negative impact of the infection on their social support; second, they gave potential reasons for negative changes.

Negative impact of HCV on social support

Deterioration of relationships: One hundred and fifty-

Table 1 Demographic and clinical characteristics of the study population

Patient characteristics	<i>n</i> (%) of total study population
Demographic characteristics	
Mean age in years (SD)	45.2 (9.2)
Women	128 (37.4)
Racial/ethnic background	
White	310 (90.6)
Black	19 (5.6)
Other ¹	13 (3.8)
Marital status	
Living with partner	212 (62.0)
Living alone	130 (38.0)
Education	
K-12	137 (40.1)
College	205 (59.5)
Employment prior to diagnosis	
Employed	296 (90.0)
Unemployed	33 (10.0)
Current employment	
Employed	217 (65.0)
Unemployed	117 (35.0)
Population of hometown	
Rural	220 (64.3)
Urban	122 (35.7)
Interview to treatment status	
Before	32 (9.4)
During	93 (27.2)
After	94 (27.5)
Naïve	123 (36.0)
Clinical characteristics	
Current substance use	
Yes	24 (7.0)
Total psychiatric diagnosis	
0	251 (73.6)
1	74 (21.7)
2	14 (4.1)
3-5	2 (0.6)
Advanced liver disease (cirrhosis)	81 (23.8)
HCV due to IV drugs	177 (62.3)
HCV due to other methods of transmission	107 (37.7)
Mean Child-Pugh score (SD)	5.46 (1.2)

SD: Standard Deviation; HCV: Hepatitis C virus; IV: Intravenous. ¹Other ethnicity includes Hispanic, Native American, Asian and Pacific Islander.

five patients (45% of the total sample) noted that having HCV had resulted in the breakdown or loss of at least one relationship. The deterioration of their social network affected sexual relationships (59 patients, 17% of the total sample) and interactions with family members (56 participants, 16% of the total sample): "I am estranged from my family. My wife and I divorced a year and a half ago. I didn't understand what was going on with me. One element of this disease is depression and an inability to think coherently sometimes. Being irrational, it's hard on relationships." Forty individuals (12% of the total sample) noted that they had experienced the loss of at least one friend.

Twenty-nine patients (8% of the total sample) mentioned multiple sources of loss: "Basically I do not have any good relationships with anyone." For these patients, the HCV diagnosis led to what they described

Table 2 Characteristics of groups categorized by social support

	Social support group		P value
	Supportive environment (n = 250)	Unsupportive environment (n = 92)	
Demographic characteristics			
Mean age in years (SD)	45.33 (9.82)	44.88 (7.52)	0.49
Women	91 (71.1)	37 (29.0)	0.52
Racial/ethnic background			0.09
White	229 (73.9)	81 (26.1)	
Black	15 (79.0)	4 (21.0)	
Other†	6 (46.2)	7 (53.8)	
Marital status			< 0.001 ¹
Living with partner	171 (80.7)	41 (19.3)	
Living alone	79 (60.8)	51 (39.2)	
Education			0.59
K-12	98 (71.5)	39 (28.5)	
College	152 (74.1)	53 (26.0)	
Employment prior to diagnosis			0.63
Employed	218 (73.6)	78 (26.4)	
Unemployed	23 (70.0)	10 (30.0)	
Current employment			0.01 ¹
Employed	169 (78.0)	48 (22.0)	
Unemployed	75 (64.1)	42 (36.0)	
Population of hometown			0.29
Rural	165 (75.0)	55 (25.0)	
Urban	85 (70.0)	37 (30.0)	
Interview to treatment status			0.03 ¹
Before	23 (72.0)	9 (28.0)	
During	72 (77.4)	21 (23.0)	
After	76 (81.0)	18 (19.1)	
Naïve	79 (64.2)	44 (36.0) ¹	
Clinical characteristics			
Current substance use			0.8
Yes	17 (71.0)	7 (29.0)	
Total psychiatric diagnosis			< 0.001 ¹
0	197 (78.5)	54 (22.0)	
1	42 (57.0)	32 (43.2)	
2	10 (71.4)	4 (29.0)	
3-5	0 (0.0)	2 (100.0)	
Advanced liver disease (cirrhosis)	63 (78.0)	18 (22.0)	0.27
HCV due to IV drugs	117 (66.0)	60 (34.0)	0.002 ¹
HCV due to other methods of transmission	89 (83.2)	18 (17.0)	
Mean Child-Pugh score (SD)			0.68
Normal	234 (69.0)	85 (25.0)	
Elevated	15 (4.4)	7 (2.1)	
Mean HAD summary scores (SD)			
Depression score	4.04 (3.47)	6.95 (4.48)	< 0.001 ¹
Anxiety score	6.57 (3.86)	8.95 (4.40)	< 0.001 ¹
Mean SIP summary scores (SD)			
Physical score	4.64 (7.03)	10.4 (12.17)	< 0.001 ¹
Psychosocial score	10.81 (13.67)	22.9 (18.82)	< 0.001 ¹

	Social support group		P value
	Supportive environment (n = 250)	Unsupportive environment (n = 92)	
Mean SIP subscale scores (SD)			
Social interaction	18.86 (25.40)	34.17 (27.72)	< 0.001 ¹
Emotional balance	8.76 (12.06)	19.3 (16.30)	< 0.001 ¹
Alertness	5.15 (8.69)	9.79 (11.24)	< 0.001 ¹
Body care and movement	7.06 (12.56)	16.09 (23.73)	0.002 ¹
Ambulation	9.60 (16.53)	22.38 (24.48)	< 0.001 ¹
Mobility	4.34 (7.67)	11.19 (13.36)	< 0.001 ¹
Communication	2.32 (6.41)	7.91 (12.63)	0.001 ¹

SD: Standard Deviation; HCV: Hepatitis C virus; IV: Intravenous; HAD: Hospital Anxiety and Depression Scale; SIP: Sickness Impact Profile. † Other ethnicity includes Hispanic, Native American, Asian and Pacific Islander. ¹Denotes statistically significant P value.

as widespread social isolation. This feeling negatively impacted nearly all aspects of their social lives: "Well, it has affected my family, like even my parents and stuff. My mother's afraid to kiss me; she thinks it's like AIDS, you know. It caused a divorce." In 25 additional cases (7% of the total sample), it was the patients themselves who chose to withdraw from families or friends: "When I first found out that I had something wrong with my liver I was very upset and didn't want anyone around me and tried to keep people from me...Right at the moment I have spent a lot of time away from my family. I'm not really wanting to go around them until I find out for sure what's going on."

Underlying reasons for a loss of social support: Three dominant themes emerged when patients explained why their social support had been lessened because of their HCV infection. (1) Transmission of the virus: First, concerns about possible transmission of the virus were described as a major stressor by 84 patients (25% of the total sample). Most of these concerns, voiced by 69 patients (20% of the total sample), focused on sexual partners and family members: "I live with a woman who's deathly afraid of contracting HCV from me, so there's friction there." A smaller number of patients (n = 36; 11% of the total sample) were afraid that they might infect friends. Because of fears related to maternal-fetal transmission, 2% of the total sample mentioned concerns about having children, which included the decision to forgo having a family: "(I feel) like I am cootied- like I have cooties. I feel like even if I wanted to have children, I couldn't." However, these often excessive concerns also led to some appropriate precautions. Forty participants (12% of the total sample) stated that they had started using various precautions, including barrier methods for contraception as well as keeping all wounds bandaged, utensils clean, and/or warning others of potential infection risks. (2) Ignorance surrounding the disease: Ignorance, sometimes leading to frank discrimination, was described by 66 participants (19% of the total sample) as directly disrupting relations with family and/or friends. One young man stated: "I lost probably half my friends as they don't know what the disease is. They just automatically put up a front. I just don't see them anymore-fear of what may happen. So ignorance is how I like to put it." In

conjunction with these feelings, 61 patients (18% of the total sample) had decided that they would not tell others of their disease to decrease the chance of experiencing a negative reaction: "I don't tell anybody 'cause...people are stupid about this disease. Just like they're stupid about AIDS." A small group of patients ($n = 11$; 3% of the total sample) did not experience discrimination, but rather sensed that those around them simply did not understand that HCV was a serious disease that could affect physical and emotional functioning: "Family members still don't understand because you don't look sick, so therefore, you should still be able to do everything." (3) Stress caused by the disease: Eighty-three participants (24% of the total sample) experienced problems in their social environment, which they attributed to disease related stress. Such HCV related stress took several forms. One was the unpredictability of the infection's outcome, which left family members and/or friends worrying about the patient: "It's sort of depressing for my family members, because they're all really concerned. I've got daughters and they're worried that I'm going to die." For some patients, a major stressor of living with HCV was their own personal concerns for family and/or friends: "I've got to make sure that my family is provided for, 'cause I don't know if I'm going to croak here anytime."

Another frequently mentioned problem with detrimental effects on relationships was fatigue ($n = 49$, 14% of the total sample). Treatment or disease related fatigue limited the patient's ability to spend time with others: "Basically I am just non-functioning; cannot maintain relationship with my wife; I cannot work. I have two little boys I am not able to spend quality time with. I can spend time with them, but...I don't have the energy to go out and play baseball with them or any of that stuff."

A final source of stress, especially for families, was the financial burden that came either through a loss of work and/or through the cost of the antiviral medication ($n = 16$, 5% of the total sample). One patient explained the reaction of her husband to the treatment of her disease: "Not good, it's a lot of strain on him and stress and he's emotionally upset because of everything. And he's had to hold the whole road as far as work and we're in debt \$40,000. You know, that's the big one, the debt and money 'cause we don't have insurance. How are we ever going to pay for it?"

DISCUSSION

This is the first analysis to display the frequency and to characterize the nature of the relationship challenges that patients with HCV experience. About one third of this large group of HCV infected patients described a negative impact of their disease on personal relationships. The most devastating consequences were with breakups or major stressors on sexual relations as experienced by about 17% of the participants, and the loss of relationships with family members in an additional 16% of cases. Our results are consistent with prior studies focusing on patients with HIV/AIDS or tuberculosis, who also felt rejected by relatives and friends^[24,25]. While all chronic diseases can

potentially tax a social support network, a diagnosis with HCV appeared to carry with it unique challenges. Patients feared interactions with others, especially sexual partners, because of the perceived risk that they might infect them. Patients also complained that those close to them lacked information about HCV, that they harbored prejudices or simply did not consider the disease to be "real" and, therefore, serious. As a result, 7% of HCV positive patients in our study personally chose to withdraw from social contact with others.

A similar breakdown occurred outside of the home, with participants reporting that friends had shied away from them. As reported in prior studies, a substantial number of patients experienced frank stigmatization^[26-29]. Changes in employment, sometimes attributed to perceived stigmatization^[10], sometimes fatigue, created additional strains, as patients were facing financial problems exacerbated by the significant costs of the antiviral treatment.

Concerns about quality of life emerged with patients' estrangement from family and friends. Relationship deterioration can constitute an important stressor, which can limit a patient's ability to cope with a disease^[30-32]. Several studies have previously found that a lack of social support and personal coping resources can affect mood and trigger depression^[33-36].

Our bivariate analysis is consistent with the above mentioned theoretical considerations. Similar to prior reports, we noted an association between emotional problems, including depression, as measured by the HAD, and lower levels of social support^[37-39]. A lower level of support was also associated with higher scores for anxiety, as measured by the HAD, more psychiatric diagnoses as listed in the medical chart, and lower psychological well-being as measured by sub-scales and summary scores of the SIP. While studies have consistently found antiviral therapy to be associated with higher levels of depression^[40-45], our cross-sectional data did not show differences in the level of social support of those currently on therapy, as compared to those who were pre or post treatment. This may be surprising at first glance because of the known effects of antiviral therapy on mood. Yet our thematic analysis pointed to specific challenges-transmission risks, unpredictable outcomes, feelings of isolation and the perceived need to withdraw from others-that are not unique to patients undergoing treatment.

Interestingly, patients with lower levels of social support were also more likely to report more severe physical symptoms, as shown in higher SIP scores on Body Care and Movement, Mobility, and Ambulation. While we did not notice a relationship with the presence of cirrhosis or the Child Pugh score, a prognostic indicator for advanced liver disease, most of the patients did not have cirrhosis or had well compensated disease, thus, limiting the discriminating value of these variables. The underlying mechanisms for the relationship between poor social support and physical functioning are unclear^[46-49]. One explanation, as noted by patients in our study, was that worse physical functioning limited their ability to meet expectations of family and/or friends.

This study was shaped by the belief that patients' accounts accurately reflected their lived experience of having HCV. As such, the narrative findings were limited to patient recall. However, we maintain that an open account by patients is the best way to understand the breadth and depth of the problems affecting their social support. In addition, we interviewed a very large group of patients with HCV, enabling us to see which themes appeared most dominant, thereby decreasing the anecdotal nature of most qualitative findings.

The study was also cross-sectional in nature, thereby limiting causal evidence of whether social support difficulties actually resulted in worse mental and physical health. Future studies should assess patient's social support level prior to HCV testing in order to determine whether the knowledge of having the disease results in negative interpersonal consequences or whether patients with risk factors for HCV are instead prone to having social difficulties. Finally, the study population was representative of the non-urban Midwest of America. Our age and sex distribution are similar to those reported in many of the prominent studies on HCV^[50-53]. Also, our results found no significant association between social support and rural/urban living or ethnicity. However, as the majority of our participants were Caucasians, the study is not an ideal vehicle for examining the role of race or ethnicity in social support.

In conclusion, as far as we are aware, our results are the first to characterize in a large patient sample the negative consequences of carrying a diagnosis of HCV on patients' social support. Providers and case managers need to be aware of the themes expressed in this analysis, as fears of contamination, feelings of isolation or withdrawal from others may reduce the emotional willingness of patients to initiate and remain on antiviral therapy. Structured educational sessions for both the patient and their partners and families could help reduce the misunderstandings expressed here, such as the ignorance of the disease or the view that the disease is somehow not "real," which can further burden the resilience of patients with HCV to cope with their disease.

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APPENDIX

- 1 Can you tell me what your condition is exactly? What physical limitations do you have and when were you initially diagnosed?
- 2 Could you tell us a little bit about yourself: the things you've done, your roles in life, who you are? What in your life are you most proud of?
- 3 Can you share with us anything specific that might have caused or helped cause your illness?
- 4 Have any of your family members had similar health problems? Does this family history worry you?
- 5 What impact has your physical condition had on the quality of your life? Would you say that your quality of life as compared with 1 year ago is better, about the same, or worse?
- 6 As you go through this experience, have you begun to think about yourself differently?
- 7 What has been the hardest thing for you to cope with related to this experience? How do you go about coping with your condition?
- 8 How does this illness make you feel emotionally? If there was one emotion that you would use to express how you feel, what would that be?
- 9 Since you were diagnosed, what would you say has been your biggest regret? Would you say you feel emotionally worse since you were diagnosed, about the same as before, or emotionally better off than before?
- 10 Can you share what health related worries you have? Do you worry more about your health than you do other aspects of your life? Did you share these worries with your doctor or nurse?
- 11 How would you describe your outlook or thoughts as you look to the future?
- 12 What are the various things that make your life meaningful for you? What do you turn to when you are in need of strength?

- 13 **We are interested in the impact this illness has had on your relationships:**
(1) If you were in a crisis, who would you turn to?
(2) Do you have a spouse or significant other?
How has that person reacted to your situation of being ill? When you think about when you were healthy, would you describe the two of you as closer, somehow further part, or just as close as you always were? **(3) Can you describe the level of support you can find in your family? Can you explain why are you able or not able to rely on your family to help you through difficult times?** **(4) Would you say that you are close to others outside of your family? Why would you say that? What about co-workers, are they understanding and supportive?**
- 14 **Have you encountered any negative judgments from others because of your disease? If yes, can you say who has made you feel this way and under what circumstances?**
- 15 How has your disease affected your sense of control in your life? What are the situations where you feel you're not in control?
- 16 What has been your experience with your doctors here or elsewhere? Would you describe it as positive or negative and what has made it positive or negative? What in your eyes makes a good doctor?
- 17 Do you feel that you can ask your doctor or nurse any question? Do you worry that you may be taking their time with your concerns?
- 18 Where do you get most of your medical information? Is there a source you tend to rely or believe in the most? In general, are there other things about your health condition that you would like to learn from your doctor?
- 19 What is the most difficult part of following the treatment the doctor recommended for you? Can you describe situations where you are unable to follow the doctor's treatment, such as taking your medicines, watching your diet, or exercising?
- 20 Did you feel comfortable with your treatment decisions? Do you feel you had choices in your treatment and that they were respected? Who did you discuss your treatment decisions with; what really helped you to make up your mind?
- 21 Do you use other or supplemental, herbal, or alternative medicines, and if yes, what are those?
- 22 If you were to describe yourself as something- it can be anything in this world--an object, an animal, anything-- before when you were healthy and now, what two things would you choose?
- 23 Is there anything else that you would like to add that would help us to understand your experience?
- 24 Finally, is there anything you would like us to share with a social worker, a pastor, or your health care team about the way you are feeling?
- Interviewing Question Guideline (Bold indicates primary questions used in this analysis)

S- Editor Wang J L- Editor Zhu LH E- Editor Ma WH

Differential effects on apoptosis induction in hepatocyte lines by stable expression of hepatitis B virus X protein

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or after induction of endogenous p53 by UV irradiation. Repression by endogenous p53 was partially reversible by stably expressed HBx in both cell lines.

CONCLUSION: Stable expression of HBx leads to deregulation of apoptosis induced by UV irradiation depending on the cell line used. In an immortalized hepatocyte line HBx acted anti-apoptotic whereas expression in a carcinoma derived hepatocyte line HBx enhanced apoptosis.

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Key words: Apoptosis; Hepatitis B virus; Hepatocyte lines

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Abstract

AIM: Hepatitis B virus protein X (HBx) has been shown to be weakly oncogenic *in vitro*. The transforming activities of HBx have been linked with the inhibition of several functions of the tumor suppressor p53. We have studied whether HBx may have different effects on p53 depending on the cell type.

METHODS: We used the human hepatoma cell line HepG2 and the immortalized murine hepatocyte line AML12 and analyzed stably transfected clones which expressed physiological amounts of HBx. P53 was induced by UV irradiation.

RESULTS: The p53 induction by UV irradiation was unaffected by stable expression of HBx. However, the expression of the cyclin kinase inhibitor p21^{waf/cip/sdi} which gets activated by p53 was affected in the HBx transformed cell line AML12-HBx9, but not in HepG2. In AML-HBx9 cells, p21^{waf/cip/sdi}-protein expression and p21^{waf/cip/sdi} transcription were deregulated. Furthermore, the process of apoptosis was affected in opposite ways in the two cell lines investigated. While stable expression of HBx enhanced apoptosis induced by UV irradiation in HepG2-cells, apoptosis was decreased in HBx transformed AML12-HBx9. P53 repressed transcription from the HBV enhancer I, when expressed from expression vectors

INTRODUCTION

The epidemiological association between chronic HBV infection and the development of hepatocellular carcinoma is well established^[1]. However, the molecular mechanism of transformation by HBV is unsolved. Among many potential factors, a viral regulatory protein named Hepatitis B virus protein X (HBx) is believed to contribute to oncogenesis in conjunction with other mechanisms. Stable expression of HBx showed a weak transforming effect in cell lines of liver origin^[2-5], in murine fibroblasts^[6,7] and in rat fibroblasts^[8-12]. Neither the molecular actions set in motion by HBx during the process of transformation, nor the role of HBx for HBV replication are well understood. HBx is dispensable for replication *in vitro*^[13], but under certain conditions HBx can enhance replication *in vitro*^[14-16]. In the woodchuck, a related virus of the same family of viruses, the hepadnaviridae^[17], the analog of HBx is essential for the establishment of infection^[18,19]. A plethora of *in vitro* activities and interactions with cellular partners by HBx have been reported^[22,23].

In the development of cancer, the delicate balance between cell proliferation and programmed cell death is often disturbed^[24]. Several functions of HBx are linked to transformation: HBx transactivates the transcription of cellular proto-oncogenes^[25-27], and is able to override cell

cycle check points^[28,29]. As the oncoproteins of many tumor viruses influence the course of apoptosis in order to transform their target cells^[30-32], several reports described the influence of stable and transient transfection of HBV products on apoptosis. Transfection of replication competent constructs of HBV^[33] and the closely related hepadnavirus woodchuck hepatitis virus^[34] enhanced apoptosis induction. This pro-apoptotic activity of hepadnavirus genomes was dependent on the integrity of the HBx-ORF^[33,34]. Several groups described a pro-apoptotic activity of HBx after transient and stable transfection^[33,34-48] whereas other groups described anti-apoptotic effects of HBx^[49-59].

The cellular tumor suppressor protein p53 is one of the key players in the induction of apoptosis after genotoxic events^[60,61]. Thus, besides influencing programmed cell death the transforming proteins of DNA tumor viruses target and inactivate p53 in quite diverse and elaborate ways^[31,32]. Several lines of evidence connect HBx with a disturbance of p53 functions. HBx binds to p53 *in vitro*^[62,63], the intracellular distribution of p53 appears to be affected by HBx^[4,64,65], p53-dependent and independent DNA repair functions are affected by HBx^[66-70]. Furthermore, HBx relieves the repression of transcription by the tumor suppressor p53 in transient reporter gene assays^[71-73], and influences the expression of the p53 induced cyclin kinase inhibitor p21^{waf/cip/sdi}^[74].

However, most of the experiments in these reports were done in transient transfection or even cell free *in vitro* systems. Because the number of HBx molecules in naturally infected cells is assumed to be low^[75-77] and transient transfections of HBx expression constructs yield very high amounts of HBx protein, we established cell lines stably expressing low amounts of HBx. Next, we studied the effect of HBx expression on several functions of p53 induced by stimuli that were designed to yield physiological p53 amounts in our cell lines. Using these *bona fide* natural conditions we were able to find that even low levels of HBx altered or even antagonized p53 functions.

MATERIALS AND METHODS

Cell lines

AML12: The highly differentiated murine hepatocyte line AML12^[78] derived from mice transgenic for TGF α can be transformed by HBx^[3]. AML12 clones stably transfected with and transformed by HBx and the control cell lines stably transfected with pSV2neo were obtained from N. Fausto. AML12 clones were cultured in Dulbecco's MEM/Nutrient Mix F12 supplemented with 10% FCS, 0.1 μ M Dexamethason, and ITS (Insulin, Transferrin, and Selene, 5 μ g/mL each).

HepG2: The differentiated but already transformed human hepatoblastoma line HepG2^[79] was cultivated in Mixed Medium/10% FCS.

CAT assay

For CAT assays, the cells per well were transfected with Lipofectamine (Life Technologies, Karlsruhe). Detection of CAT protein was done with the CAT-ELISA Kit

(Roche, Mannheim) according to the instructions of the manufacturer.

UV irradiation

All cell lines were plated out one day before irradiation. Directly before irradiation, the medium was removed and the cultures were washed with PBS. UV irradiation was done in the UV-Stratalinker (Stratagene, Leimen, Germany) at 254 nm. The cells were exposed to UV after removing the cover of the dishes.

For induction of apoptosis, 1000 cells were plated in triplicates on culture dishes with a diameter of 95 mm. After irradiation with the indicated energy in the stratalinker, the cell cultures were grown for 5-15 d, until colonies visible by eye were detected. After washing with PBS, the colonies were stained and fixed with 1% crystal violet in 20% methanol for 5 min. After washing with H₂O, the surviving colonies were counted. Each experiment was done in triplicate and repeated at least thrice.

Western blot

For the lysis of cells, 450 μ L of ice cold lysis buffer were added to each well of a six-well plate for 30 min on ice. The lysis was based on RIPA-buffer (0.01 mol/L Tris-HCl, pH 7.4; 0.15 mol/L NaCl; 1% (w/v) sodium desoxycholate; 0.1% (w/v) SDS; 1% Triton X-100) supplemented with Leupeptin and Aprotinin. To digest disturbing chromosomal DNA 250 U/mL Benzonase (Novagen, Merck, Darmstadt) was added to the lysis buffer. After the incubation period, the lysate was cleared by centrifugation at 20 000 g for 5 min. The protein content of the lysates was determined using the BCA assay and equal amounts of protein (20-50 μ g) were separated on an SDS gel. After blotting and incubation with the appropriate primary and secondary antibodies, the membranes were developed using Chemiluminescence Blotting Substrate from Boehringer Mannheim, Germany according to the manufacturer's instructions.

Antibodies

The following antibodies were used for the detection of the indicated proteins: murine p53: AB7 (biotinylated polyclonal sheep antibodies from Oncogene Science, via Dianova, Hamburg, Germany); human p53: mAb PAb1801 (Pharmlingen, Hamburg, Germany); murine p21: mAb AB4 (OP76 Oncogene, via Dianova, Hamburg, Germany); human p21: p21 (187) (sc-817), St Cruz Biotechnology.

As secondary antibody, we used POD-labeled anti-murine IgG from donkey (Jackson ImmunoResearch, via Dianova, Hamburg, Germany) or POD-streptavidin (Calbiochem, Bad Soden, Germany) for murine p53.

Northern blot

For Northern blot, total cellular RNA was extracted from the cells using TRIzol (Gibco). Twenty micrograms of DNase I digested RNA was separated on a glyoxal agarose gel (1% in 10 mmol/L Na₂HPO₄) and blotted onto positively charged nylon membrane. The blot was hybridized with a ³²P labeled p21-specific probe derived from pCMVp21 (murine).

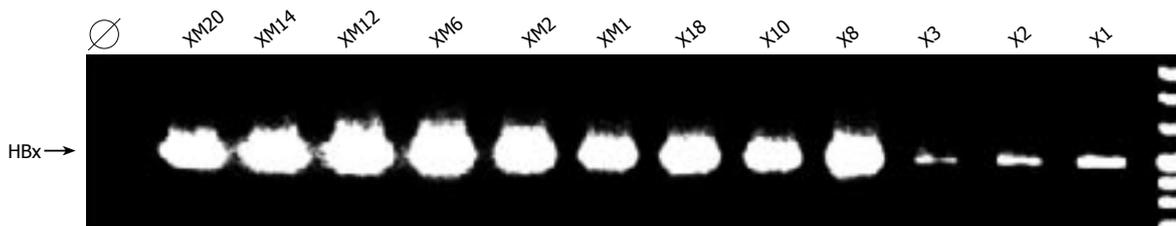


Figure 1 RT-PCR for the detection of HBx-RNA in HepG2 cells stably transfected HBx-expression constructs. Total cellular RNA from clones obtained after stable transfection with expression vector pRCCMVX for wt-HBx (X1, X2, X3, X8, X10, and X18) or for inactive HBx (pRCCMVXM, XM1, XM2, XM6, XM12, XM14, and XM20) was amplified by RT-PCR with primers used for cloning the complete HBx-ORF.

Ribonucleotide protection assay (RPA)

For RPA, an RNA probe complementary to human p21 was transcribed with Maxiscript, Ambion from the construct pCMVsd^[80] according to the instructions of the manufacturer. As an internal control, we used actin transcribed from pTRI- β -Actin (Ambion, Austin, TX, USA).

The RNA probe was purified by electrophoresis in 5% acrylamide/8 mol/L urea gel. The probe (0.5–2 μ g) was labeled with biotin using the BrightStarTM Psoralen-Biotin kit (Ambion, Austin, TX, USA).

The RPA was performed with the HybSpeedTM RPA kit from (Ambion, Austin, TX, USA). After digestion of the samples as described in the manual of the kit, the samples were run on 5% PAGE/8 mol/L urea in TBE and blotted onto a positively charged nylon membrane (Amersham, Braunschweig). Signal detection was performed with streptavidin labeled with alkaline phosphatase using the BrightStarTM BioDetectTM kit from Ambion, Austin, TX, USA.

RT-PCR

For RT-PCR, 2 μ g of total cellular RNA extracted with Qiagen RNeasy was digested with DNase I for 45 min. One microgram of DNase I digested RNA was reverse transcribed with superscript (Gibco). One quarter of the reverse transcribed RNA was amplified with Taq polymerase (1' 94°C, 1' 54°C, 1' 72°C: \times 35 cycles) using HBx-specific primers, which had been used for cloning the complete HBx ORF into pRcCMV.

Constructs

pRcCMVX: The complete HBx ORF of an HBV genotype A genome (serotype adw2; isolate 991; EMBL accession no.: X51970) was cloned with the help of PCR into the *EcoRI* and *XbaI*-site of pCDNA3, Invitrogen, and controlled by sequencing.

pRcCMVXM: Identical to pRcCMVX except for two stop codons introduced by a G to A exchange of nt 1443 (GAA \rightarrow TAA)^[2] and a C to A exchange of nt 1684 (TCA \rightarrow TAA). Thus, translation of full-length HBx is stopped by exchange of nt 1443 after 23 aa and expression of presumed small HBx proteins is made impossible by exchange of nt 1684, which introduces a stop directly after the third start codon in frame in the HBx-ORF.

PS9: HBV enhancer I (nt 1040–1374 from EMBL accession no.: X51970) was cloned into pBLCAT3^[81].

pC53SN3: Expression vector for wt-p53^[82]. P53 is

expressed from the CMV enhancer in the vector pCMV.

pcDNA3p21 (murine): Murine p21 was cloned by RT-PCR with primers waf-AS and waf-S^[33] from total cellular RNA derived from AML12 cells. After *EcoRI* digestion, the amplicon was cloned into pCDNA3. Sequencing gave complete identity with murine p21 from GenBank.

RESULTS

Establishing HBx-expressing HepG2-cell lines

HepG2 cells—a completely transformed hepatoma cell line—were stably transfected with the HBx-expression construct pRcCMVX. As a negative control, HepG2 cells were transfected with the isogenic HBx protein expression-negative construct pRcCMVXM. Clones obtained after G418 treatment were analyzed for the expression of HBx-RNA expression by RT-PCR (Figure 1). 11/13 wt-HBx transfected and 11/11 HBxM transfected clones expressed HBx RNA detectable by RT-PCR. While all HBxM transfected clones expressed similar amounts of HBx RNA as judged by RT-PCR, it appeared as if HBx RNA expression in wt-HBx transfected clones varied significantly (Figure 1). This reminds of the findings in stably HBx transfected clones of rat fibroblast origin, where HBx expression was downregulated in most clones^[33].

Clone HepG2X8 expressing wt-HBx and control clone HepG2 XM2 transfected with the HBx-expression minus construct pRcCMVXM expressed similar amounts of HBx-RNA and were chosen to be studied in detail.

In comparison, we analyzed the immortalized murine hepatocyte line AML12^[78] and the AML12-clone HBx9 transformed by HBx^[3].

Analysis of HBx functions in HBx-expressing AML12 and HepG2 clones

One well-characterized function of HBx is its potential to transactivate virtually any promoter or enhancer. We were unable to detect HBx protein in stably transfected AML12 and HepG2 clones (data not shown and Ref. [3])—a problem encountered by many other groups. In order to show the presence of active HBx, we analyzed whether transcriptional activation by HBx could be detected in cell lines stably expressing HBx RNA. Indeed, the expression from HBV enhancer I was approximately sevenfold stronger in the HBx expressing cell line HepG2-X8 than in the control cell line HepG2-XM2 (Figure 2B). In AML12 cell transactivation by HBx was lower (Figure 2A).

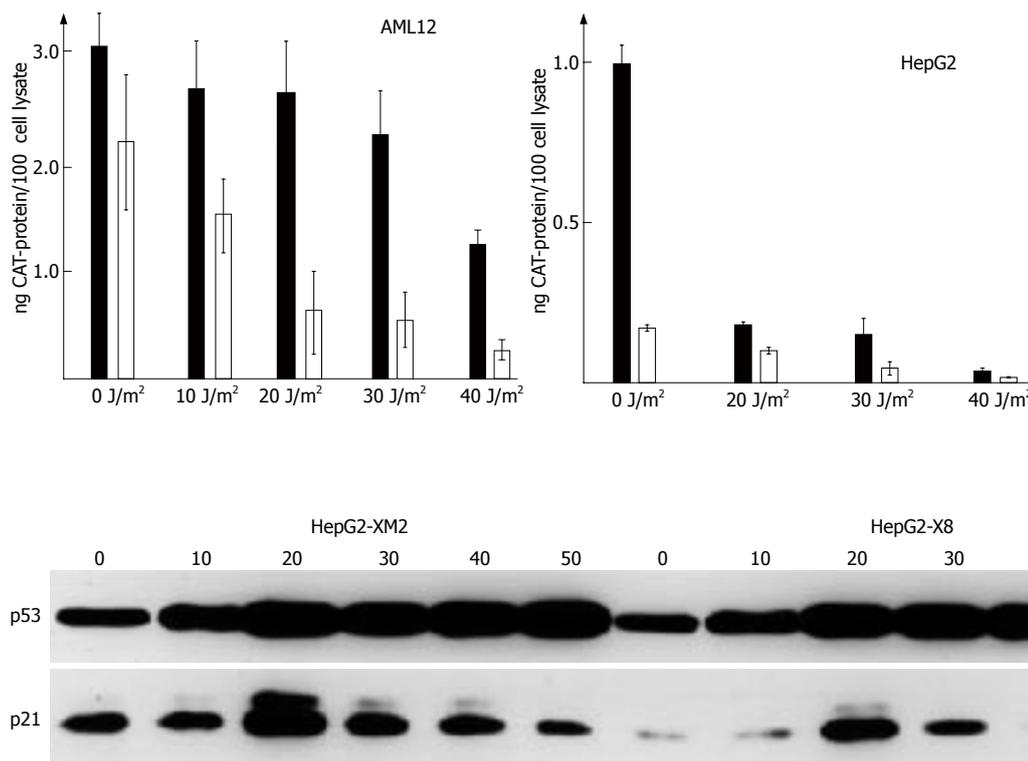


Figure 2 HBx activates the HBV-Enhancer I *in vivo* and can partially counteract a repression by p53 induced by UV irradiation. CAT expression driven by Enhancer I of HBV is indicated as bars. AML12 (A) or HepG2 (B) derived cell lines expressing HBx (HepG2-X8 or AML12-HBx9) are indicated in black and the controls which did not express functional HBx (AML12 or HepG2-XM2) are shown in white bars. The cell lines were transfected with a CAT-expression vector driven by enhancer I of HBV in triplicate. Four hours after transfection, the cultures were irradiated with the indicated dose of UV. The lysis of the cells was done 18 h post irradiation.

Figure 3 Induction of p53- and p21^{waf/cip/sdi}-protein expression in HepG2-derived cell lines after irradiation with UV by immune blot. The dose of UV used for irradiation is indicated. The cells were lysed 18 h after irradiation with UV.

Table 1 Survival of HepG2- and AML12-derived HBx-expressing and control cell lines after irradiation with UV

	0 J/m ²	20 J/m ²	50 J/m ²
HepG2-XM2	100 ± 1.4	17.7 ± 8.0	1.0 ± 0.9
HepG2-X8	100 ± 20.9	1.1 ± 0.6	0.2 ± 0.1
HepG2-XM2	100 ± 11.8	15.8 ± 7.1	1.38 ± 1.5
HepG2-X8	100 ± 20.3	2.7 ± 1.5	0.0
HepG2-XM2	100 ± 15.6	13.5 ± 6.9	3.7 ± 0.8
HepG2-X8	100 ± 7.4	6.8 ± 0.5	1.7 ± 1.2
AML12	100 ± 5.1	4.3 ± 0.6	1.1 ± 1.0
AML12-HBx9	100 ± 14.2	13.5 ± 8.9	6.0 ± 2.2
AML12	100 ± 27.2	17.0 ± 6.3	0.6 ± 0.04
AML12-HBx9	100 ± 3.8	28.0 ± 7.0	6.8 ± 2.4
AML12	100 ± 4.4	11.0 ± 4.1	0.4 ± 0.5
AML12-HBx9	100 ± 4.6	17 ± 5.1	3.4 ± 1.8

The percentage of cells surviving UV-treatment of three independent experiments. For higher reproducibility the HBx-expressing and the control cell lines were irradiated in one batch.

HBx acts pro-apoptotic in HepG2- and anti-apoptotic in AML12-cell lines

To analyze the extent of apoptosis quantitatively, we investigated the survival of the cell lines under investigation after UV irradiation. Five hundred cells in triplicate were plated and irradiated with the dose indicated. Surviving colonies were counted after about 14 d. Table 1 shows that stable expression of HBx acts pro-apoptotic in HepG2-derived and anti-apoptotic in AML12-derived cell lines. The pro-apoptotic effect of HBx in HepG2-X8 was most obvious in cell lines irradiated with 20 J/m².

For example in one experiment 17.7% ± 8.0 cells of the control cell line HepG2-XM2 survived irradiation with 20 J/m², whereas only 1.1% ± 0.6 of identically treated HBx expressing HepG2-X8 cells were able to grow out to visible colonies. AML12 cells were more sensitive against UV irradiation. Expression of HBx acted anti-apoptotic in the HBx expressing AML12 cell line and enhanced the survival rates to similar levels as in HepG2XM control cells.

No influence of HBx on induction of p53 in HepG2- and in AML12-cell lines

p53 is a central protein in the induction of apoptosis^[83]. After many sublethal stimuli, a complex set of events takes place that results in an increased expression of p53 in the nucleus. Thus, we analyzed by immune blots of whole cell extracts whether HBx was able to modulate expression of p53 after UV irradiation of the cell lines. Irradiation with increasing doses (0-50 J/m²) of UV led to enhanced expression of p53 in control and HBx expressing cell lines (Figures 3 and 4). HBx had no effect on the induction of p53 in cell lines of AML12 and HepG2 origin. p53 expression was easily detectable after irradiation with 20 J/m² and maximal expression was reached after irradiation with 40-50 J/m² in the HepG2 and AML12 cell lines investigated (Figures 3 and 4). These data are in accordance with reports on p53 expression in HepG2-cells^[84], human^[85], and murine^[85,86] fibroblasts and primary rat keratinocytes^[87].

We also analyzed the kinetics of p53 induction after UV irradiation with 40 J/m². p53 levels increased after 1 h to reach a maximum 18 h after irradiation. Thereafter,

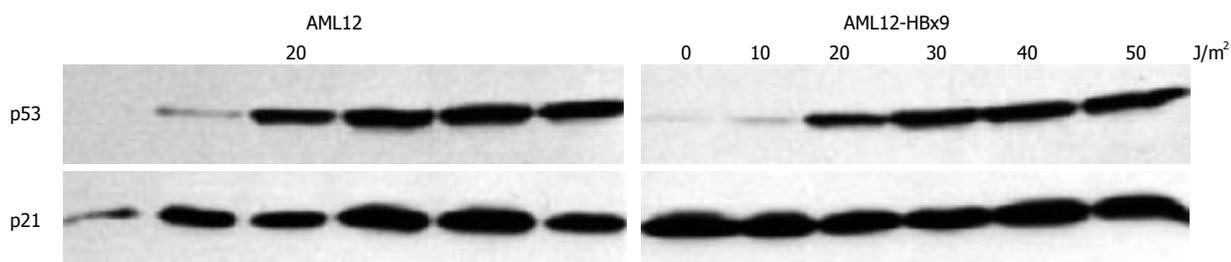


Figure 4 Induction of p53- and p21^{waf/cip/sdi}-protein expression in AML12-derived cell lines after irradiation with UV by immune blot. The dose of UV used for irradiation is indicated. The cells were lysed 18 h after irradiation with UV.

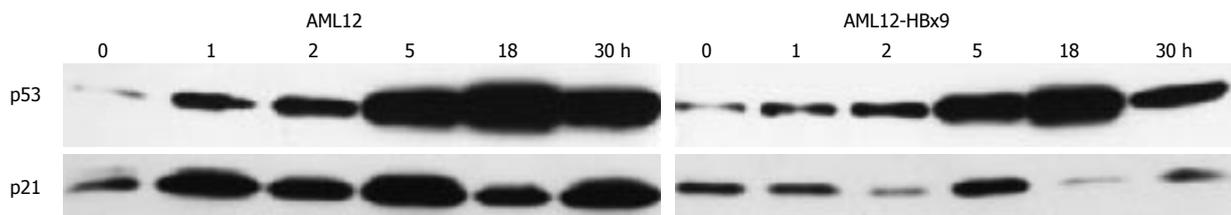


Figure 5 Kinetics of induced p53- and p21^{waf/cip/sdi}-protein expression after irradiation with UV by immune blot in AML12 cell lines. The time in hours after irradiation with 40 J/m² UV is indicated.

p53 expression decreased (Figure 5). Again, HBx had no influence on the kinetics of p53 induction after UV irradiation.

HBx counteracts a repression by UV irradiation *in vivo*

We next asked whether HBx may influence functions of p53. One extensively investigated activity of p53 is the ability to repress the transcription of many genes^[88]. Thus, we analyzed whether expression of the reporter gene CAT from HBV enhancer I is repressed by p53 - or other factors activated after induction of programmed cell death - *in vivo*. Irradiation of HepG2 and AML12-cell lines with increasing doses of UV caused indeed a strong repression of expression from HBV enhancer I (Figures 2A and B). The repression was highest at 40 J/m² (Figures 2A and B), the UV dose leading to maximum expression of p53 (Figures 2 and 3). However, a strong reduction of CAT-repression could already be observed at the lowest UV dose of 10 J/m² administered. Interestingly, in the tumor cell line HepG2 (Figure 2B), UV irradiation caused a much stronger repression of expression from enhancer I than in AML12 cells (Figure 2A).

In HBx expressing cell lines, the UV-induced repression was partially counteracted. This anti-repressive activity of HBx was strongest in AML12-HBx9 and still significant in HepG2 cells (Figures 2A and B). In the AML-cell line HBx9 irradiated with 40 J/m² the CAT-expression was 4.9-fold higher than in the parental cell line AML12 without HBx. Thus, HBx seems to be able to alleviate transcriptional repression by p53.

Induction of p53 and p21^{waf/cip/sdi} by UV irradiation

Because we observed that HBx influenced the extent of transcriptional repression by p53 we also wanted to analyze if HBx had any influence on transcriptional activation by p53. After UV irradiation, p53 is stabilized^[89] and acts as a

transcription factor by binding to the promoter of certain target genes leading to enhanced transcription from these promoters, for example of the cyclin kinase inhibitor p21^{waf/cip/sdi}^[90]. Thus, after UV irradiation, the expression of p21^{waf/cip/sdi} is enhanced^[86,91]. We therefore analyzed if stable expression of HBx influenced p53's transcriptional activation of the endogenous p21^{waf/cip/sdi}-promoter. UV irradiation of the cell lines with increasing doses of UV led to an increase of p21^{waf/cip/sdi} protein expression (Figures 3 and 4). As reported by other groups, irradiation with high doses of 40-50 J/m² UV again caused a decline of the induced p21^{waf/cip/sdi} protein expression^[86,91]. An analysis of the temporal regulation of p21^{waf/cip/sdi} protein expression (Figure 5) after UV irradiation in AML12 cells showed an undulation of p21^{waf/cip/sdi} protein expression in the parental cell line and the HBx transformed clone 9. A similar phenomenon was described in the mouse liver after 70% partial hepatectomy^[92]. However, these authors analyzed RNA expression and different time points. Interestingly, stable expression of HBx in HepG2 led to a lower expression of p21^{waf/cip/sdi}-protein. However, in the HBx transformed cell line AML12-HBx9, the level of p21^{waf/cip/sdi}-protein expression after UV irradiation changed from experiment to experiment and no reproducible data for p21 could be obtained, although p53-expression from identical blots (upper lane) gave reproducible results (Figures 4 and 5). It appeared as if the expression of p21^{waf/cip/sdi}-protein was deregulated in a seemingly random way by HBx because p21^{waf/cip/sdi}-protein expression was reproducible in the parental cell line (Figures 4 and 5).

Butz *et al* had described an uncoupling of the regularly inducible p21^{waf/cip/sdi}-RNA expression from the deregulated p21^{waf/cip/sdi}-protein expression upon genotoxic stress in some cell lines^[109]. Thus, we analyzed the induction of p21^{waf/cip/sdi}-RNA in the HBx transfected cell lines. Indeed, analysis of p21^{waf/cip/sdi}-RNA expression in the hepatocyte

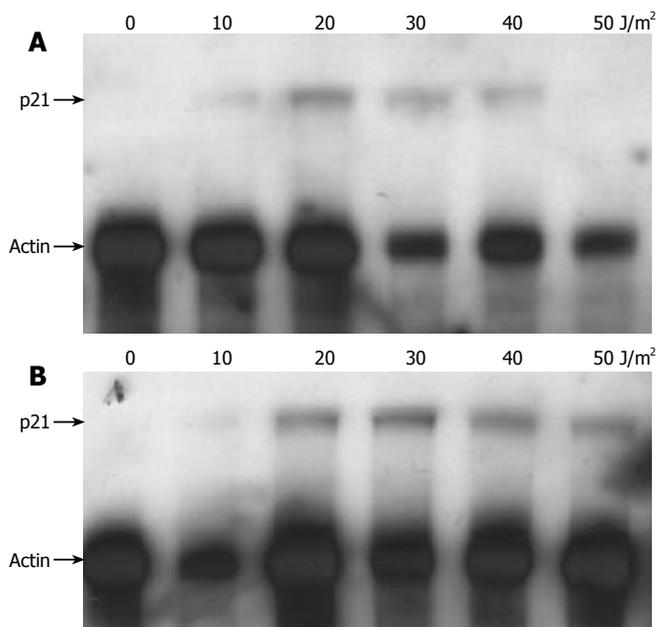


Figure 6 Detection of p21^{waf/cip/sdi}-RNA by ribonuclease protection assay in the cell line HepG2-XM2 (A) and -XB8 (B). Total cellular RNA was isolated from cell cultures irradiated with the indicated dose of UV 18 h after irradiation. As internal control actin was detected by RPA.

lines under investigation gave reproducible results. Irradiation with UV increased the expression of p21^{waf/cip/sdi}-RNA in the HBx-expressing cell lines HepG2-X8 and the control cell lines HepG2-XM2 and AML12 to reach a peak at 20-30 J/m² to decrease thereafter (Figures 6 and 7). Analysis of p21^{waf/cip/sdi}-RNA expression in the HBx-expressing clone AML12-HBx9 - in contrast to analysis of p21^{waf/cip/sdi}-protein expression - gave reproducible results. However, no decline of p21^{waf/cip/sdi}-RNA expression was observed at higher doses of UV irradiation as with the other three cell lines under investigation (Figure 7). Quite in contrast, in the HBx-expressing clone AML12-HBx9 the amount of p21^{waf/cip/sdi}-RNA increased continuously even at the highest dose of UV irradiation of 50 UV J/m². Thus, HBx strongly influenced the regulation of p21^{waf/cip/sdi} expression in the HBx-transformed cell line AML12-HBx9.

CONCLUSION

One of the most important functions of p53 is the induction of programmed cell death in order to maintain the integrity of the genome by inhibiting the survival of cells with damaged DNA^[93]. To analyze the integrity of p53 function in cell lines stably expressing HBx RNA, we investigated several p53 activities during induction of apoptosis by UV. Irradiation with 20 J/m² quite uniformly induced massive cell death in all cell lines analyzed. The cell death was due to apoptosis because we observed nucleosomal DNA fragmentation in all cell lines under investigation (data not shown).

However, we observed that induction of apoptosis was enhanced in HBx expressing cell lines of HepG2-origin and reduced in HBx expressing cell lines of AML12-origin. These opposing effects of HBx are in agreement with the

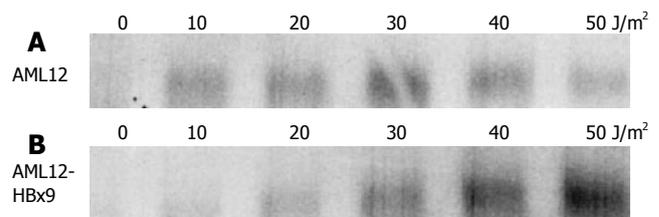


Figure 7 Detection of p21^{waf/cip/sdi}-RNA by northern blot in the cell line AML12 (A) and the HBx-transformed clone AML12-HBx9 (B). Total cellular RNA was isolated from cell cultures irradiated with the indicated dose of UV 18 h after irradiation.

published results on the influence of HBx on programmed cell death. Several groups described a pro-apoptotic activity of HBx after transient and stable transfection^[33,34-48] whereas other groups described anti-apoptotic effects of HBx^[49-59].

We thus set out to study key events in the induction of apoptosis after UV irradiation. We investigated whether HBx had an effect on the induction of p53 in our cell lines. No evidence was found that HBx influenced the induction of p53 by UV. Neither p53's expression by stabilization of p53 as reported for adenovirus E1A or the large T-Antigen of SV40^[94] was increased, nor was p53's expression decreased in the presence of HBx as described for E6 of the papilloma viruses^[95]. Our results agree with reports from Refs. [85] and [97] who also did not find altered p53 induction or expression in HBV expressing HepG2 cells.

A widely accepted hypothesis assumes that a higher expression of the cyclin kinase inhibitor p21^{waf/cip/sdi} after DNA damage gives an advantage for survival because cells arrest in G₁ and gain time to repair DNA damage instead of succumbing to apoptosis^[88]. DNA damage induced in cell lines with a targeted deletion of the p21^{waf/cip/sdi}-gene led to programmed cell death, whereas the wt-cell line survived in cell cycle arrest^[97]. After induction, p53 binds as a transcription factor to the promoter of p21^{waf/cip/sdi} and increases its transcription^[90]. Our results on induction of p21^{waf/cip/sdi}-protein and RNA by UV irradiation in the control cell lines (AML12 and HepG2-XM2) are in accordance with data from literature^[86,87,91]. Interestingly, the HBx-expressing clone HepG2-clone X8 expressed less p21^{waf/cip/sdi} than the control. A decrease as well as an increase of p21^{waf/cip/sdi} expression in response to HBV infection has been described in literature. In microarrays, an enhanced expression of p21^{waf/cip/sdi} was seen in Hep3B and HepG2 lines stably expressing HBx^[96,98] and in the HBV expressing cell line HepG2.2.15^[99], decreased expression of p21^{waf/cip/sdi} was seen in HBV associated HCC^[100], in transiently transfected primary human hepatocytes^[101] and in two liver samples from patients with chronic HBV infection^[101].

The reason for the differential effect of HBx on the expression of p21^{waf/cip/sdi} in AML12 and HepG2 cell lines is unknown. However, transduction of HBx into primary human hepatocytes and stably transformed hepatocyte lines had reciprocal effects on the expression of several target genes^[101]. It appears possible that stable expression of HBx in the immortalized AML12 cell line mimics the situation found in acutely infected liver

with deregulated and in some circumstances enhanced p21^{waf/cip/sdi} expression^[100], whereas stable expression of HBx in hepatoma cell line HepG2 with slightly decreased expression of p21^{waf/cip/sdi} mirrors the situation found in HBV associated HCC^[100], i.e. in the endpoint of hepatocarcinogenesis.

It appears possible that HBx transactivates the enhancer of p21^{waf/cip/sdi} in AML12 cells directly, as has been reported for other cell lines^[74,96]. In addition, HBx may enhance the physiological activation of p21^{waf/cip/sdi} by p53. Haviv *et al.*^[102] have shown that HBx can drastically enhance p53 mediated transcriptional activation. In consequence, HBx expressing AML12 clones would express more p21^{waf/cip/sdi} than controls. Furthermore, HBx may have a stronger effect on the activation of the transcription factor *ets* in AML12 cells than in HepG2 cells, as *ets* has been suggested to play a crucial role in the transactivation of the p21^{waf/cip/sdi} enhancer by HBx^[96]. These assumptions would be in agreement with the higher and deregulated expression of p21^{waf/cip/sdi}-RNA in the HBx expressing AML12 cell line. An enhanced expression of p21^{waf/cip/sdi} in the HBx transformed cell line AML12-HBx9 reminds of findings in HTLV-1 transformed cell lines^[103,104]. Tax, the transforming protein of HTLV-1 virus^[105] has many functional similarities with HBx^[106]. The oncoprotein E7 of the papilloma viruses also enhances the expression of p21^{waf/cip/sdi}^[107,108]. As described for otherwise unrelated cell lines^[109], we also observed that the expression of p21^{waf/cip/sdi}-protein was deregulated and did not seem to correlate with p21^{waf/cip/sdi}-RNA-expression in HBx-expressing cell line AML12-HBx9 (Figures 4, 5 and 7).

Our results indicate that HBx is able to partially relieve repression of expression from the HBV enhancer I by physiological amounts of p53 as observed after induction by UV irradiation (Figure 2). Other groups reported complete relief of transcriptional repression by p53 after transient transfection of HBx^[71] or HBV-dimers^[72] or in *in vitro* transcription assays using *in vitro* translated p53 and HBx proteins^[73]. However, transient transfection of HBx expression constructs leads to high expression of HBx, whereas stable expression of HBx as in our experiments only leads to low level expression of HBx that may be more comparable to the natural situation in tissue of chronically infected individuals where detection of HBx is rather difficult because of its low level of expression^[75-77]. Moreover, in transient assays using expression vectors for p53 and HBx, a complete relief of transcriptional repression by p53 was only seen when 100-fold more HBx expression vector than p53 was used^[71]. When equal amounts of expression constructs were used, HBx was not able to relieve transcriptional repression by p53^[71,110]. Our results indicate that stable expression of HBx in hepatocyte lines antagonizes functions of physiological doses of p53 induced by UV and confirm the results of transient transfections^[71,72].

In summary, we found several p53 linked activities that were altered in cell lines stably expressing HBx. By use of conditions which closely match the natural situation, we were able to show that HBx is able to alleviate some of the p53 induced effects taking place after the induction of apoptosis by UV irradiation. The differences we observe

between the HBx expressing immortalized hepatocyte line AML12 and the hepatoma cell line HepG2 are due to a differential activation of intracellular signal transduction pathways - as found in inducible HBx expressing AML12-clones^[5,111] - and will be the subject of further investigations.

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Virological course of hepatitis A virus as determined by real time RT-PCR: Correlation with biochemical, immunological and genotypic profiles

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Abstract

AIM: To undertake analysis of hepatitis A viral load, alanine aminotransferase (ALT), and viral genotypes with duration of viremia, and to correlate these parameters with CD4⁺/CD8⁺ lymphocyte populations that control cell-mediated immunity.

METHODS: Cell counts were carried out using fresh whole blood collected in EDTA vials using a fluorescence activated cell sorter. Hepatitis A virus (HAV) RNA was extracted from blood serum, reverse transcribed into cDNA and quantified by Real-Time polymerase chain reaction and was genotyped.

RESULTS: Among 11 patients, 10 could be analyzed completely. Of these, 3 had severe acute hepatitis (s-AH) and the remainder had a self-limited acute hepatitis A (AHA), with one patient with fulminant disease (encephalopathy Grade IV) dying on the 4th d. The ALT level was significantly higher both in AHA (1070.9 ± 894.3; $P = 0.0014$) and s-AH (1713.9 ± 886.3; $P = 0.001$) compared to normal controls (23.6 ± 7.2). The prothrombin time in s-AH patients (21.0 ± 2.0; $P = 0.02$) was significantly higher than in AHA (14.3 ± 1.1;

$P = 0.44$). The CD4⁺/CD8⁺ ratio in AHA patients (1.17 ± 0.11; $P = 0.22$) and s-AH (0.83 ± 0.12; $P = 0.0002$) were lower than seen in normal healthy controls (1.52). Self-limited cases had peak viral load at the beginning of analysis while in s-AH patients this occurred at the 15th or 30th d. In acute and severe groups, one patient each belonged to genotype IA, with the remaining 8 cases belonging to genotype IIIA. The only fulminant hepatic failure case belonged to genotype IA. HAV viral load and ALT values collected during the entire course of the self-limited infection were directly correlated but this was not the case for s-AH patients.

CONCLUSION: Based on a small-scale study, the persistently higher viral load of s-AH might be due to diminished cellular immunity and hemolysis. The duration of viremia was dependent on the host, as the viral genotype had no apparent role in clinical outcome of AVH and s-AH cases.

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Key words: Viral load; Real-time PCR; Immunological response; Severe acute hepatitis; Self-limited acute hepatitis

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INTRODUCTION

Hepatitis A virus (HAV) is a common cause of acute viral infections in humans^[1]. It is a ubiquitous virus readily transmitted by the feco-oral route^[2-5]. Nucleotide sequence analysis of HAV has classified the virus in seven different genotypes, which include human (I, II, III, and VII) and simian (IV, V, and VI) genotypes^[6]. HAV infection follows a benign course; it is often asymptomatic in younger children, but can develop into a fatal fulminant form or severe acute hepatitis in older persons^[7-9].

To date, however, there is limited knowledge of viral load, or the length of viral persistence both in the blood circulation and in fecal excretion. It has been reported that a relapse may occur 30-90 d after the initial onset of the disease^[10-12] and virus has been detected in the stool of patients^[13]. Recently, outbreaks of HAV have occurred among hemophiliacs receiving organic solvent and detergent-treated factor VIII, a fact that stresses the potential usefulness of a reliable and widely applicable technique for quantifying viral load in blood samples^[14-18]. The level and the length of HAV viremia involve the additional risk of the carrier becoming an infectious source of hepatitis A^[19].

We undertook further examination of hepatitis A viremia during the course of infection to understand whether viral load was correlated with cell-mediated immunity. The pathogenetic mechanisms underlying hepatocellular injury in acute hepatitis are poorly understood^[20]. There is general agreement that HAV infection does not evolve to chronic hepatitis in man^[21], and immune mechanisms have been suspected of playing a major role in eliminating virus-infected liver cells^[22-24]. The aim of this study was to undertake analysis of HAV viral load, alanine aminotransferase, and viral genotypes with the duration of viremia, and to correlate these parameters with populations of CD4⁺/CD8⁺ lymphocytes that controls cell-mediated immunity.

MATERIALS AND METHODS

Patients and blood samples

Patients attending the Medical Out Patient Department of Lok Nayak Hospital, New Delhi, with the characteristic symptoms of acute viral hepatitis such as jaundice, fever, general malaise, fatigue, nausea, vomiting, anorexia and right upper quadrant discomfort, were screened for the study. Ten mL of blood were collected by venipuncture from those patients, who gave consent for five different visits. The study was approved by the ethical committee of Maulana Azad Medical College, as per the Declaration of Helsinki (1995). Consecutive blood samples were collected from 10 acute hepatitis A patients on the 0th, 7th, 15th, 30th and 45th d between July 2004 and June 2005. No sample was collected before the onset of symptoms. The '0th' d was defined as the first day when the patient presented after the onset of jaundice. Ten healthy subjects who had no evidence of liver disease or dysfunction were taken as control.

Serological tests

Laboratory examination of alanine aminotransferase (ALT), aspartate aminotransferase (AST), prothrombin time (PT), and total bilirubin levels were carried out by standard methods. IgM anti-HAV were detected by ELISA (HAVAB-MEIA); Abbott Laboratories, North Chicago, IL), IgM anti-HEV (Qiagen, Hilden, Germany), HBsAg (Qiagen, Hilden, Germany) and anti-HCV (Bio-Rad, San Francisco, CA, USA) were measured according to the manufacturer's protocol.

FACS analysis of T-lymphocyte profile

One milliliter whole blood was collected into a vial

containing EDTA and was employed for CD4⁺ (T helper) and CD8⁺ (T suppressor) cell counts within 24 h of collection, using a Fluorescence Activated Cell Sorter (FACS) (Becton Dickenson Electronics Laboratory, Mountain View, California). This system quantifies CD4⁺, CD8⁺ and CD3⁺ T lymphocytes as absolute numbers of lymphocytes per μL (mm^3) of blood, and the CD4⁺/CD8⁺ T lymphocyte ratio. Samples from healthy controls and patients were also run for cell counts using the manufacturer's protocol and reagents.

Primers, probe and standard for real-time amplification

HAV RNA was extracted using the QIAampTM viral RNA extraction kit (Qiagen, Germany). Viral RNA was amplified using primers derived from the most constant region, the 5' non-coding region (5' NCR)^[25,26]. The primers used were, forward primer HAV1 (22: 5'-TTT CCG GAG CCC CTC TTG-3'), as wild type (M14707) reverse primers HAV2 (85: 5'-AAA GGG AAA TTT AGC CTA TAG CC-3') and HAV3 (85: 5'-AAA GGG AAA ATT TAG CCT ATA GCC-3'), and HAV-probe (58: 5'-FAM-ACT TGA TAC CTC ACC GCC GTT TGC CT-TAMRA-3') and RNA standard representing the 5'NCR region was constructed according to Costa-Mattioli *et al*^[27].

Fluorogenic quantitative Real-Time PCR and direct sequencing

RT-PCR was carried out with a HAV quantification kit (Roche Diagnostics GmbH, Germany) according to the manufacturer's instructions. The total volume of the reaction mixture was 25 μL (15 μL of mastermix with 10 μL of the RNA template) in 0.2 mL tubes. The capillaries were sealed, centrifuged, and transferred to the Rotor Gene 3000 real-time PCR machine (Corbett Research, Sydney, Australia). Reverse-transcription was done for 15 min at 50°C followed by 5 min denaturation at 95°C. The corresponding cDNA's were amplified by PCR (20 s at 95°C, 30 s at 50°C acquiring FAM, and 20 s at 72°C) over 45 cycles, and an 87 bp fragment was obtained. The CT values from the clinical samples were plotted on the standard curve, and the number of copies was calculated automatically.

PCR amplification from part of the VP1/2A region of HAV genome was directly sequenced for genotyping^[28-30]. Sequencing was done with an ABI Prism 310 Genetic Analyzer (ABI, Foster City, CA). Sequencing analysis was performed using ClustalW and the phylogenetic inference by version 1.81 of the PHYLIP software package (Professor J. Felsenstein, Department of Genetics, University of Washington, Washington, DC).

Statistical analysis

All data were analyzed by two tailed tests, and a *P* value less than 0.05 was considered significant. We used chi-square test and student's t-test as appropriate.

RESULTS

Comparison of clinical features between AVH and s-AH groups

The average age of all patients was 20.8 ± 15.5 years

Table 1 Clinical, biochemical and immunological characterization of different groups (AVH, s-AH, and normal control)

Characteristic	AVH ¹	s-AH ²	Normal Control ³
No. of cases	07	03	10
Sex (M/F)	3/4	3/0	7/3
Age (mean ± SD)	0.4 ± 17.9	27.7 ± 2.08	26.2 ± 3.6
ALT (mean ± SD; IU/L)	1070.9 ± 894.3	1713.9 ± 886.3	23.6 ± 7.2
AST (mean ± SD; IU/L)	621.3 ± 242.8	1614 ± 234.7	23.8 ± 5.8
T. Bilirubin (mean ± SD; mg/dL)	9.8 ± 3.8	24.5 ± 3.0	0.74 ± 0.43
Prothrombin Time (Seconds)	14.3 ± 1.1	21.0 ± 2.0	12.5 ± 0.5
Mean CD4 ⁺ /CD8 ⁺	1.17 ± 0.11	0.83 ± 0.12	1.52 ± 0.11

ALT: AVH vs s-AH $P = 0.29$ (not significant); AVH vs NC $P = 0.0014$ (highly significant); s-AH vs NC $P = 0.001$ (highly significant); **AST:** AVH vs s-AH $P = 0.0034$ (highly significant); AVH vs NC $P = 0.00014$ (highly significant); s-AH vs NC $P = 0.00044$ (highly significant); **T. Bil:** AVH vs s-AH $P = 0.076$ (significant); AVH vs NC $P = 0.00017$ (highly significant); s-AH vs NC $P = 0.0023$ (highly significant); **PT:** AVH vs s-AH $P = 0.11$ (not significant); AVH vs NC $P = 0.44$ (not significant); s-AH vs NC $P = 0.02$ (significant); **Mean CD4⁺/CD8⁺:** AVH vs s-AH $P = 0.46$ (not significant); AVH vs NC $P = 0.22$ (not significant); s-AH vs NC $P = 0.0002$ (highly significant). **Note:** AVH¹ (Acute viral hepatitis); s-AH² (Severe acute hepatitis); NC³ (Normal Control).

and ranged from 3 to 59 years. The average age and sex ratio of two groups of patients is shown in Table 1. 10 of 11 patients could be followed completely, of which 3 progressed to severe acute hepatitis (s-AH), which is defined on the basis of a prothrombin time (PT) < 40% of normal range. The remainder of patients had self-limited acute hepatitis A (AHA) and one died of fulminant hepatic failure (Grade IV encephalopathy) at the 4th d of follow up. There was no difference between the groups with respect to clinical symptoms, such as flu-like prodromes (including arthralgia, or headache), fever, nausea, vomiting, abdominal pain, pruritus, and diarrhea.

Comparison of biochemical features between AVH and s-AH groups

In Figure 1, the time course of viral load and serum transaminase ALT levels is presented for all patients. ALT values for both groups followed a decreasing trend towards normal from the initial to final day of follow up. The mean liver function profile of s-AH patients was higher compared to the AVH cases as shown in Table 1. The mean prothrombin time (PT) of s-AH patients was (21.0 ± 2.0 ; $P = 0.02$) significantly higher than that of acute cases (14.3 ± 1.1 ; $P = 0.44$).

Comparison of immunological profiles between AVH and s-AH groups

The mean immunological (CD4⁺/CD8⁺) ratio in patients with acute viral hepatitis A was (1.17 ± 0.1) higher than that in the severe acute cases (0.83 ± 0.12). As shown in Table 1, the CD4⁺/CD8⁺ ratio in normal controls (NC) (1.52 ± 0.11) ratio was almost twice as high as in s-AH ($P = 0.0002$). There was no significant decrease in the immunological ratio of AVH cases compared to normal controls.

Comparison of viral load between AVH and s-AH groups

In the acute case, Ind-301, the viral load on the initial day was 4.5×10^5 , and decreased further to $> 10^3$ copies/mL. As shown in Figure 1, Ind-303 and Ind-306 followed almost similar trends with viral loads of 2.6×10^5 and 1.2×10^5 respectively at 0th d while $< 10^2$ copies/mL still persisted at the end of follow up. The viral load of Ind-304 was 1.0×10^5 at start and complete elimination of the virus with zero copies/mL was seen at the end of follow up, as shown in Figure 1. Patients Ind-308 and Ind-310 displayed a peak viral load at 0th d like the cases described above, while Ind-309 peaked on the 15th d, but values then decreased to less than 100 copies/mL in all three. In the severe cases Ind-302 and Ind-305, the viral load on the initial day of follow up was 1.2×10^5 and 1.1×10^5 respectively, and reached a peak on the 30th d, quite different from the acute case shown in Figure 1. At the end of follow up, the viral load was significantly higher ($> 1.0 \times 10^5$) as compared to AVH. In-patient Ind-307, viral load reached the peak (4.6×10^5) at 15th d and decreased subsequently to 4.3×10^4 copies/mL at the end of the follow up.

Comparison of genotype (s) between AVH and s-AH groups

In Table 2, the maximal viral load was compared with genotyping and geographical distribution. As shown in phylogenetic tree (Figure 2), the patients Ind-301 (DQ179131) and Ind-302 (DQ179132) were categorized as genotype IA, while the remainder Ind-303 (DQ179133), Ind-304 (DQ179134), Ind-305 (DQ179135), Ind-306 (DQ179136), Ind-307 (DQ179134), Ind-308 (DQ182495), Ind-309 (DQ182496), and Ind-310 (DQ182497) were classified as the IIIA genotype. The only fulminant hepatic failure case, Ind-274 (DQ182500), belonged to genotype I A.

DISCUSSION

Hepatitis A remains the most frequent form of viral hepatitis observed in a large number of countries^[31,32]. Recent publications have demonstrated that the duration of the viremic phase is much longer than assumed^[10,27]. A serum HAV viral load assay could therefore be helpful in the management of severe hepatitis A. Real-Time reverse transcription (Rotor Gene 3000, Corbett Research, Sydney, Australia), was used for the quantitative detection of the HAV genome in human sera in individuals who displayed varying disease courses^[27,33,34]. The fluorescence signal due to the cleavage of the fluorogenic probe is generated only if the target sequence for the probe is amplified by the PCR. Therefore, no signals are generated by non-specific amplification.

The alanine aminotransferase level of AVH cases on the initial day was significantly higher ($\geq 10^3$) and the decreased upon subsequent follow up, which corresponds to earlier findings that demonstrate a direct correlation of viral load with serum ALT^[27,35]. In our study, the mean prothrombin time in AVH was not higher while severe cases showed significant elevations compared to normal controls. This could be due to anemia (hemolysis) as this

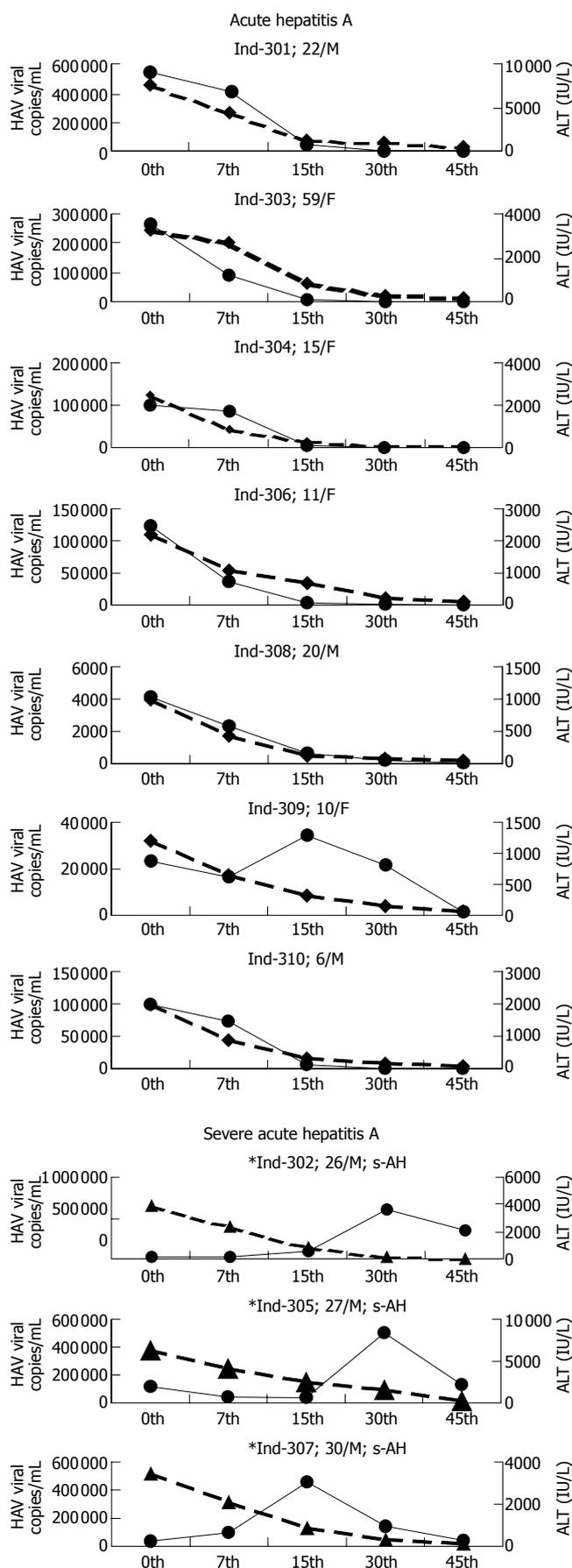


Figure 1 Relationship of hepatitis A virus (HAV) viral load (●) to levels of alanine aminotransferase (ALT) (▲) in serum at different day of follow up.

Table 2 Comparison of patient's genotypes to viral load and geographical distribution

Patients	Genotype	Maximal viral load Copies/mL	Geographical distribution
Ind-301	I A	4.5×10^5	New Delhi
¹ Ind-302	I A	6.0×10^5	New Delhi
Ind-303	III A	2.6×10^5	Uttar Pradesh
Ind-304	III A	1.0×10^5	Delhi
¹ Ind-305	III A	5.0×10^5	Delhi
Ind-306	III A	1.2×10^5	New Delhi
¹ Ind-307	III A	4.6×10^5	Uttar Pradesh
Ind-308	III A	4.1×10^3	New Delhi
Ind-309	III A	3.4×10^4	Haryana
Ind-310	III A	9.8×10^4	New Delhi

¹Represents severe acute hepatitis.

is not uncommon during viral hepatitis^[36]. We excluded glucose-6 phosphate dehydrogenase (G6PD) deficiency as a cause of hemolysis and anemia in our cases^[36,37].

The immunological changes in acute cases was not significantly different from those in severe hepatitis A. The lymphocyte ratio (mean $CD4^+/CD8^+$) of the severe patients was, however, significantly lower, which could be due to diminished cellular immunity as compared to the normal controls. Previous studies have also shown that generation of $CD4^+$ T cells in the thymus is severely impeded as either a direct or indirect consequence of active viral replication^[35,38]. We speculate that severe HAV infection may be triggered by diminished cellular immunity in susceptible patients, which may have increased the liver damage due to hepatitis A.

On the basis of our results, genome quantities measured on the first (0th) d of clinical diagnosis in HAV infected humans reached peaked copies/mL in acute viral hepatitis and attained normality towards the end of follow up. The kinetics of peak viral load attainment in s-AH was quite different from that in the acute self-limited cases since at the end of follow up high copies/mL still persisted, as shown in Figure 1. The acute results confirm the levels recently estimated by Chudy *et al*^[15]. The progression of severity due to diminished cellular immunity and hemolysis might be directly linked to high viral persistence throughout the follow up.

Most of the patients examined during acute self-limited illness belonged to genotype IIIA, other than Ind-301 who belonged to IA^[6]. Among the severe cases, Ind-302 belonged to IA while other two belonged to IIIA, which means genotypic variations likely do not play a crucial role in determination of the viral load as described earlier by Normann^[35]. The only FHF case, who died at the 4th d of follow up, belonged to genotype IA. The question arises whether the duration of viremia is dependent on the genotype, or the immunological and/or biochemical profile. Our results showed that the duration of the viremia was dependent on the host, as the viral genotype had no role in acute self-limited illness and severe acute hepatitis A cases. This differs considerably from earlier

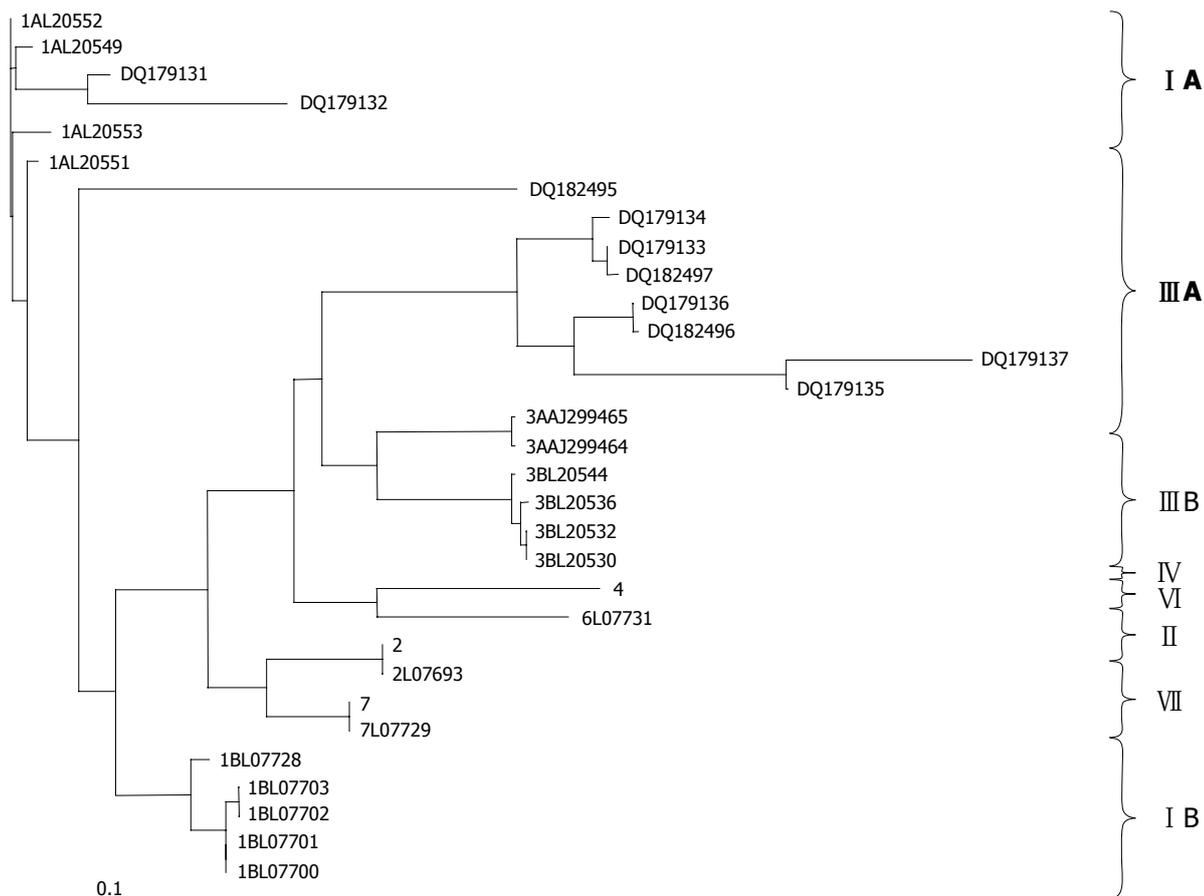


Figure 2 A neighbor-joining phylogenetic tree for isolates of hepatitis A virus based on the sequencing of the VP1-P2A region. Isolates DQ179131 (Ind-301), DQ179132 (Ind-302), DQ179133 (Ind-303), DQ179134 (Ind-304), DQ179135 (Ind-305), DQ179136 (Ind-306), DQ179137 (Ind-307), DQ182495 (Ind-308), DQ182496 (Ind-309), DQ182497 (Ind-310) were collected during the present study, in PCR Hepatitis Laboratory, MAM College and associated LNJ Hospital, New Delhi, India.

findings which showed that a long duration of viremia was found in patients infected with HAV genotype IA^[35].

In conclusion, HAV viral load and alanine aminotransferase (ALT) values collected during the entire course of a self-limited acute infection were directly correlated, but this was not found in s-AH cases. The duration of viremia was dependent on the host (biochemical and immunological profiles), as the viral genotype had no role in the various groups studied. The mean prothrombin time in severe acute hepatitis was higher than seen in acute self-limited illness. The immunological (CD4⁺/CD8⁺) ratio of s-AH was quite low compared to the acute self-limited illness, and s-AH patients showed diminished cellular immunity and complications. There was no difference in the final clinical outcome and recovery of liver function was seen in all patients. The limitation of the study is that the number of patients examined was relatively small. Therefore, there is a need for further research on the duration and magnitude of HAV viremia in a large cohort of human patients to properly document complications and management.

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***H pylori* receptor MHC class II contributes to the dynamic gastric epithelial apoptotic response**

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the accessory molecule FADD, and this delay in apoptosis induction could allow for prolonged cytokine secretion by *H pylori*-infected gastric epithelial cells.

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Key words: *H pylori*; Epithelium; Apoptosis; Class II MHC

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Abstract

AIM: To investigate the role of MHC class II in the modulation of gastric epithelial cell apoptosis induced by *H pylori* infection.

METHODS: After stimulating a human gastric epithelial cell line with bacteria or agonist antibodies specific for MHC class II and CD95, the quantitation of apoptotic and anti-apoptotic events, including caspase activation, BCL-2 activation, and FADD recruitment, was performed with a fluorometric assay, a cytometric bead array, and confocal microscopy, respectively.

RESULTS: Pretreatment of N87 cells with the anti-MHC class II IgM antibody RFD1 resulted in a reduction in global caspase activation at 24 h of *H pylori* infection. When caspase 3 activation was specifically measured, crosslinking of MHC class II resulted in markedly reduced caspase activation, while simple ligation of MHC class II did not. Crosslinking of MHC class II also resulted in an increased activation of the anti-apoptosis molecule BCL-2 compared to simple ligation. Confocal microscope analysis demonstrated that the pretreatment of gastric epithelial cells with a crosslinking anti-MHC class II IgM blocked the recruitment of FADD to the cell surface.

CONCLUSION: The ability of MHC class II to modulate gastric epithelial apoptosis is at least partially dependent on its crosslinking. The crosslinking of this molecule has anti-apoptotic effects during the earlier time points of *H pylori* infection. This effect is possibly mediated by the ability of MHC class II to modulate the activation of the pro-apoptotic receptor Fas by blocking the recruitment of

INTRODUCTION

H pylori infects over half of the people in the world. Seropositivity may reach 80%-100% in underdeveloped nations. This gram negative bacterium is a major contributor to chronic gastritis and peptic ulcer formation, and is strongly associated with gastric carcinoma and lymphoma^[1,2]. Gastric carcinoma remains the second most deadly form of cancer^[3]. While much is known about the clinical manifestations of *H pylori* infection, how this pathogen manipulates gastric epithelial cells in the host to its advantage are unknown. Previous reports by our group have demonstrated that MHC class II expressed on the surface of gastric epithelial cells serve as a receptor for *H pylori*^[4,5]. A potential consequence of bacterial interaction with MHC class II proteins is the subsequent crosslinking of these molecules. This may impact cellular responses key to the initiation and propagation of *H pylori* pathogenesis that results in tissue damage of the gastro-duodenal mucosa.

One such clinically significant cellular response to *H pylori* infection is apoptosis. The induction of apoptosis in MHC class II⁺ host cells able to direct the immune response would represent a mechanism by which the bacteria could impair local antigen presentation to T cells. Furthermore, induction of apoptosis would cause "leakiness" of the epithelium, leading to inflammation that could upregulate the expression of *H pylori* receptors on surrounding cells. For example, IFN γ , an inflammatory cytokine produced by CD4⁺ T cells within the infected gastric mucosa, upregulates class II MHC expression in gastric epithelial cells. However, uncontrolled epithelial apoptosis would

quickly lead to the destruction of *H pylori*'s niche within the gastric mucosa. Thus, mechanisms by which these bacteria could moderate the host apoptotic response must also be considered.

Previous reports show that CD95 (Fas) plays an important role in *H pylori*-mediated apoptosis of the gastric epithelium^{16,71}. Once Fas is activated on the cell surface, the FADD (Fas associated death domain) protein is recruited to the cytoplasmic domains of the trimerized Fas on the plasma membrane. FADD is then responsible for the activation of caspase 8. However, the interaction between *H pylori* receptors and pro-apoptotic death receptors such as Fas has not been well investigated. Combined with our previous data demonstrating the role of MHC class II in *H pylori* binding to gastric epithelial cells (GEC), it might be suggested that the complex dynamics regulating apoptosis during infection might be due to either complementary or antagonistic interactions between multiple signaling receptors on the cell surface. Furthermore, the possibility that MHC class II crosslinking modulates pro-death accessory molecules within the cytoplasm must also be investigated.

MATERIALS AND METHODS

Cell and Bacterial Culture

The human gastric epithelial cell line N87 was obtained from ATCC and cultured in RPMI containing 100 mL/L fetal calf serum and supplemented with glutamine. *H pylori* cag+ clinical isolate LC-11¹⁸¹ was grown on blood agar base (Becton Dickinson) at 37°C under microaerobic conditions and harvested into Brucella broth containing 100 mL/L fetal bovine serum. Bacteria in broth were rocked gently overnight at 37°C under microaerobic conditions prior to centrifugation. *H pylori* was resuspended in PBS and concentration was determined by absorbance at 530 nm using a spectrophotometer ($1 A = 2 \times 10^8$ cfu/mL) (DU-65 Becton Dickinson Instruments, Fullerton, CA).

Antibodies

Monoclonal anti-human MHC class II IgM (clone RFD1) was obtained from Serotec, Raleigh, NC. Monoclonal IgM antibody against CD-95 (clone IPO-4) used to induce apoptosis was obtained from Kamiya Biomedical Co., Seattle, WA. The hybridomas secreting anti-human MHC class II IVA-12 and L243 (mIgG) were obtained from ATCC and were used to produce ascites fluid in mice and the antibodies were purified with a protein G column. Anti-human CD95-PE was obtained from Becton Dickinson/Pharmingen, San Jose, CA. Alexa-conjugated secondary antibodies were obtained from Molecular Probes Inc., Eugene, OR.

Global Caspase Activation Assay

The global (non-specific) activation of caspases in our cell line was quantified using the Homogeneous Caspase Activation kit from Roche Applied Science, Indianapolis, IN. Cells were grown in serum containing media in 96-well plates at a seeding density of 10^4 cells/well for 18 h prior to treatment. After treatment, the media was aspirated and

a substrate-containing lysis solution was applied to the cells. After a 2 h incubation at 37°C, the cleaved substrate product resulting from the action of activated caspases was quantitated using a fluorometer ($\lambda = 521$ nm).

Caspase 3 and BCL-2 activation

Caspase 3 activation and BCL-2 expression were quantitated using the Human Apoptosis Cytometric Bead Array kit from Becton Dickinson, Franklin Lakes, NJ. The experiments were conducted according to the kit instructions. Briefly, bead populations with distinct fluorescent intensities were coated with antibodies specific for activated caspase 3 and BCL-2. The capture beads, sample lysates, and PE-conjugated detection reagent were incubated together to form sandwich complexes. After washing, the beads were run through a flow cytometer to generate MFI data, which was then analyzed with Becton Dickinson CBA Analysis Software. Sample data was normalized with specific protein standards to provide quantification of the proteins of interest.

Confocal microscopy

Cells were grown on Collagen I-coated tissue culture inserts (BD Biosciences) to 50%-75% confluency. After cell permeabilization and intracellular staining, the inserts were mounted on glass slides with coverslips. Images were obtained on a Zeiss LSM510 META advanced laser scanning confocal microscope (LSCM). Approximately 50 separate images were obtained at 0.5-0.6 micron increments for X-Z axis reconstruction.

Statistical analysis

Data is presented as the mean \pm SE and analyzed using Student's *t* test. Significance is defined as $P < 0.05$.

RESULTS

Global Caspase Activation Assay

To determine the effect of MHC class II crosslinking at the cell surface on the activation of caspases, we cultured the gastric epithelial cell line N87 in the presence of 100 kU/L IFN γ for 48 h to induce the upregulation of surface MHC class II. Cells were then rested in serum containing medium alone for 2 d prior to bacterial and antibody treatment. Cells pretreated with antibody were exposed to RFD1 (10 mg/L) for 30 min prior to the addition of *H pylori*. Camptothecin (10 μ md/L) was used as a positive control for caspase activation and an isotype antibody, which had no effect on the cells, was another control. After 24 h of infection, crosslinking MHC class II prior to the addition of *H pylori* significantly ($P < 0.05$) reduced global caspase activation compared to *H pylori* infected samples with no pretreatment (Figure 1).

Apoptosis Cytometric Bead Array

Caspase 3 is an effector caspase whose activation is central in the apoptosis pathway triggered by Fas trimerization. To specifically examine the activation of caspase 3 as a result of MHC class II ligation versus crosslinking, we used a cytometric bead array that incorporates antibodies

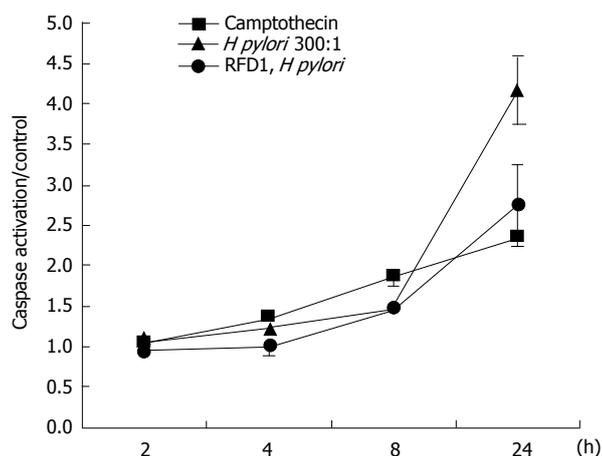


Figure 1 Total caspase activation detected using a colorimetric assay. (means of the treatments / control (untreated) samples).

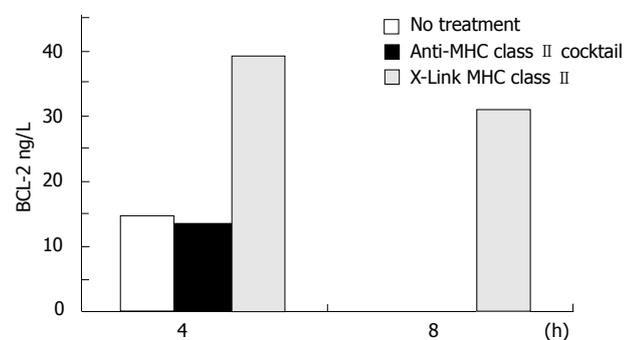


Figure 3 The effects of ligating vs crosslinking N87 cell surface MHC class II molecules on BCL-2 expression. Using the apoptosis cytometric bead array kit, beads exposed to the samples were run through a flow cytometer to obtain MFI data. These results were normalized to BCL-2 standards to give values expressed as ng/L of BCL-2.

to the activated form of caspase 3. At 4 and 8 h treatment of IFN γ -treated N87 cells, crosslinking MHC class II with biotinylated anti-MHC class II IgG antibodies L243 and IVA12 resulted in a reduced activation of caspase 3 compared to ligating MHC class II with unbiotinylated cocktail. After 4 h of treatment, ligation of MHC class II resulted in a 103% increase in caspase 3 activation compared to a 33% increase after crosslinking. At 8 h of treatment, crosslinking MHC class II resulted in a negligible increase in caspase 3 activation compared with control, while ligation with the antibody cocktail increased caspase 3 activation by 33% over control (untreated) samples (Figure 2).

The expression of the anti-apoptotic BCL-2 molecule was measured simultaneously with caspase 3 activation. As crosslinking of MHC class II resulted in reduced activation of the pro-apoptotic caspase 3 compared to MHC class II ligation, MHC class II crosslinking but not ligation produced an increase in the expression of BCL-2. MHC class II crosslinked samples showed a 169% increase over control in BCL-2 expression at 4 h treatment. After 8 h treatment, the MHC class II crosslinked samples contained over 30 ng/L of BCL-2 while the untreated and MHC class II ligated samples had no detectable BCL-2 (Figure 3).

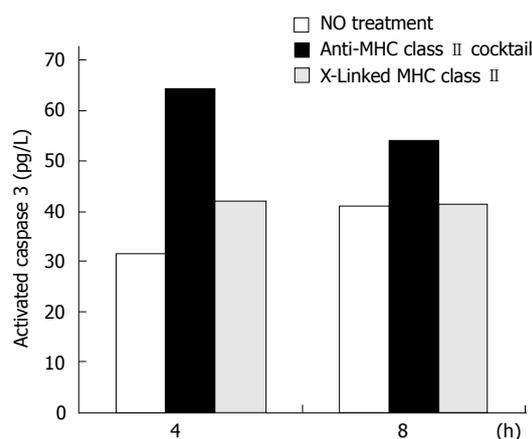


Figure 2 The effects of ligating vs crosslinking N87 cell surface MHC class II molecules on caspase 3 activation. Using the apoptosis cytometric bead array kit, beads exposed to the samples were run through a flow cytometer to obtain MFI data. These results were normalized to activated caspase 3 standards to give values expressed as pg/mL of activated caspase 3.

Confocal microscopy analysis of FADD recruitment

Fas aggregation induces the recruitment of the adapter protein Fas-associated death domain (FADD) to the cytoplasmic tail of Fas. To determine whether MHC class II crosslinking affects FADD recruitment, we did confocal microscopy in gastric epithelial cells treated with anti-Fas with and without pretreatment with anti-MHC class II. N87 cells were treated with 100 kU/L IFN γ for 48 h to increase the surface expression of MHC class II and Fas. Cells were then seeded onto filter inserts with media alone for 24 h before treatment. Prior to permeabilization, fixation, and staining, samples were A: left untreated (control), B: treated for 1 h with the anti-Fas IgM clone IPO-4 or C: pretreated with the anti-MHC class II IgM clone RFD1 for 30 min prior to adding IPO-4. After washing, the cells were then permeabilized and fixed to allow staining of intracellular FADD. The filter inserts were then mounted onto slides and immediately visualized with a confocal microscope. The large square panel represents an X-Y axis “top-down” perspective on the adherent N87 cells. The top and side rectangular panels in each figure represent X-Z axis reconstructions, which provide an elevation view of the apical and basolateral sides of the cell section (Figure 4).

DISCUSSION

Apoptosis accounts for most of the cell loss in the gastrointestinal tract^[9]. Because cellular turnover rate in the gut epithelium is so high, disturbances in the homeostasis of cell proliferation and cell death can potentially lead to disease states. If apoptosis were induced at a higher rate than new cell generation, tissue atrophy and ulceration might occur. Conversely, downregulation or blocking of apoptotic pathways might result in neoplasia. Furthermore, it is possible that hyperplasia is a response to pro-apoptotic stimuli or that increased apoptosis results from an induced hyperproliferative stimulus^[10]. These potential scenarios, and the molecular dynamics responsible

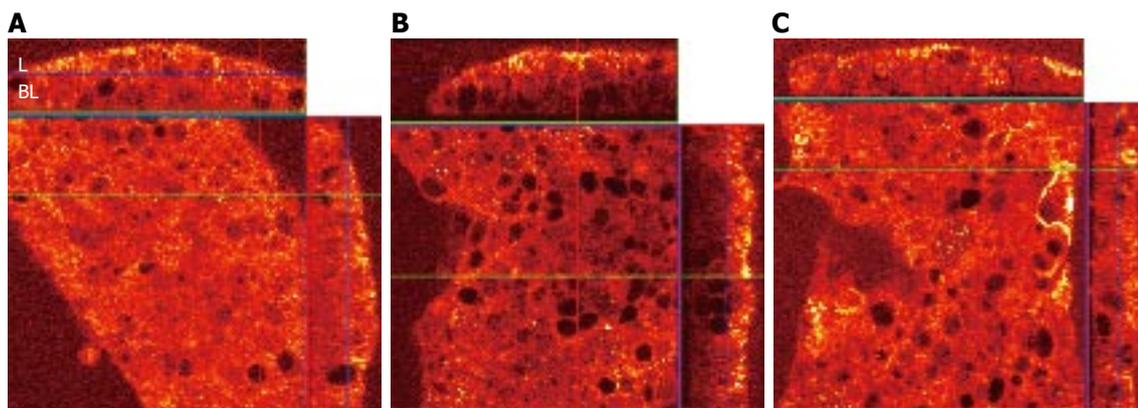


Figure 4 A: Viewing the X-Z panels, the FADD (stained red, with higher densities staining yellow) in untreated (control) cells distributed equally throughout the cytoplasm. "L" represents the apical (luminal) surface and "BL" represents the basolateral surface of the N87 monolayer; B: Labeled FADD in cells treated with the Fas trimerizing antibody is recruited to the apical surface, leaving much of the cytoplasm with reduced FADD staining; C: When the anti-MHC class II IgM RFD1 is applied to the cell monolayer 30 min prior to treatment with the Fas agonist IPO4 IgM, the recruitment of FADD to the cell surface is markedly reduced.

for them, become particularly important when considering the divergent clinical manifestations of *H pylori* infection. Two clinical observations support the concept of mutually exclusive disease states resulting from a disturbance in the delicate balance between apoptosis and proliferation of the mucosal epithelium. The first stems from multiple studies that demonstrated patients exhibiting duodenal ulcers have a lower risk of developing gastric cancer^[11,12]. The second suggests an inverse association between *H pylori* infection and esophageal adenocarcinoma^[13,14]. Although these correlations continue to be scrutinized, clearly, any factor affecting the apoptotic levels within or causing the formation of ulcerative or neoplastic epithelial tissue is critical in influencing the ultimate disease state. Therefore, the divergent and somewhat mutually exclusive nature of *H pylori* disease pathology provides a strong incentive to elucidate the epithelial receptors for *H pylori* during infection, and to investigate results of receptor ligation and crosslinking on host cell apoptosis.

The apoptosis-inducing effect of *H pylori* on gastric epithelial cells has been thoroughly demonstrated^[4-7,10,15-18]. Furthermore, our group's findings demonstrated that MHC class II not only binds *H pylori*, but also can initiate signals affecting apoptotic pathways. It suggests an important role for this molecule in influencing *H pylori* pathogenesis. The literature offers conflicting reports on the apoptotic *vs* anti-apoptotic effects of MHC class II binding. More specifically, there are opposing reports on the effect of MHC class II ligation on the Fas death pathway in mouse and human B cells^[19,20]. Previous studies from our group have revealed that signals induced by the long term (72 h) crosslinking of MHC class II are pro-apoptotic^[4,5]. However, the results we have reported here indicate that crosslinking of MHC class II can induce anti-apoptotic effects at time points less than 24 h. Furthermore, we have demonstrated the ability of MHC class II crosslinking to inhibit a key component of the Fas death pathway. Our findings that MHC class II crosslinking inhibits *H pylori*-induced caspase activation, that ligation *vs* crosslinking of MHC class II has differential effects on apoptotic-associated molecules, and that MHC class II crosslinking

inhibits FADD recruitment are intriguing because of their potential role in the divergent pathophysiologic host response to this human pathogen. By acting as a bacterial receptor, as well as modulating the important apoptotic processes during infection, MHC class II becomes a critically important molecule in the context of *H pylori* pathogenesis. Nonetheless, it is important to continue to study all GEC surface molecules that have the potential to influence the signal events initiated by *H pylori* binding. There is significant evidence that other surface molecules in addition to MHC class II are capable of binding *H pylori*; there have been numerous studies demonstrating the role of Lewis b (Le^b) blood group antigen in *H pylori* adherence to the epithelium^[22,23]. More recently, it has been suggested that the dimeric form of the trefoil protein TFF1 avidly binds *H pylori*^[24]. The implications of each of these findings in the physical interaction between bacterium and host is important because of the need to understand the molecules responsible for bacterial adhesion. They are also important because of the possibility that a particular bacterial receptor influences the downstream signal transduction events initiated when *H pylori* binds to a secondary receptor, or activates a death receptor.

Deciphering the complexity of *H pylori* pathogenesis in order to reduce its contribution to human gastric disease will require continued investigation into the interactions between bacterial receptors and their effects on the host epithelial cell homeostasis.

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S- Editor Pan BR L- Editor Zhao JB E- Editor Bai SH

BASIC RESEARCH

Effects of Ca²⁺ channel blockers on store-operated Ca²⁺ channel currents of Kupffer cells after hepatic ischemia/reperfusion injury in rats

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have obviously protective effects on I/R injury, probably by inhibiting Isoc in Kupffer cells and preventing the activation of Kupffer cells.

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Key words: Kupffer cell; Ischemia/reperfusion; Store-operated calcium channel currents; 2-aminoethoxydiphenyl borate; SK&F96365; Econazole; Miconazole

Jiang N, Zhang ZM, Liu L, Zhang C, Zhang YL, Zhang ZC. Effects of Ca²⁺ channel blockers on store-operated Ca²⁺ channel currents of Kupffer cells after hepatic ischemia/reperfusion injury in rats. *World J Gastroenterol* 2006; 12(29): 4694-4698

<http://www.wjgnet.com/1007-9327/12/4694.asp>

Abstract

AIM: To study the effects of hepatic ischemia/reperfusion (I/R) injury on store-operated calcium channel (SOC) currents (I_{soc}) in freshly isolated rat Kupffer cells, and the effects of Ca²⁺ channel blockers, 2-aminoethoxydiphenyl borate (2-APB), SK&F96365, econazole and miconazole, on I_{soc} in isolated rat Kupffer cells after hepatic I/R injury.

METHODS: The model of rat hepatic I/R injury was established. Whole-cell patch-clamp techniques were performed to investigate the effects of 2-APB, SK&F96365, econazole and miconazole on I_{soc} in isolated rat Kupffer cells after hepatic I/R injury.

RESULTS: I/R injury significantly increased I_{soc} from -80.4 ± 25.2 pA to -159.5 ± 34.5 pA ($^bP < 0.01$, $n = 30$). 2-APB (20, 40, 60, 80, 100 μ mol/L), SK&F96365 (5, 10, 20, 40, 50 μ mol/L), econazole (0.1, 0.3, 1, 3, 10 μ mol/L) and miconazole (0.1, 0.3, 1, 3, 10 μ mol/L) inhibited I_{soc} in a concentration-dependent manner with IC₅₀ of 37.41 μ mol/L ($n = 8$), 5.89 μ mol/L ($n = 11$), 0.21 μ mol/L ($n = 13$), and 0.28 μ mol/L ($n = 10$). The peak value of I_{soc} in the I-V relationship was decreased by the blockers in different concentrations, but the reverse potential of I_{soc} was not transformed.

CONCLUSION: SOC is the main channel for the influx of Ca²⁺ during hepatic I/R injuries. Calcium channel blockers, 2-APB, SK&F96365, econazole and miconazole,

INTRODUCTION

In clinical practice of hepatobiliary surgery, various factors, such as shock, inflammation, hepatic trauma, operation of liver and biliary tract (in case of necessities of interrupting hepatic portal vein), liver transplantation, are mutually related to common pathophysiological procedures, named hepatic ischemia/reperfusion (I/R) injury. Kupffer cells play an important role in hepatic I/R injury, hepatic I/R injury can be relieved when Kupffer cells are inactivated, and store-operated Ca²⁺ channels (SOC) are present both in almost all non-excitabile cells and in some excitabile cells^[1-4], but there is no report about SOC in Kupffer cells. 2-APB, SK&F96365, econazole and miconazole have been used as the blockers of SOC in many cells. In the present study, we investigated the effects of hepatic I/R injury on store-operated calcium channel currents (I_{soc}) in freshly isolated rat Kupffer cells, and the effects of calcium channel blockers, 2-APB, SK&F96365, econazole and miconazole on I_{soc} in isolated rat Kupffer cells after hepatic I/R injury.

MATERIALS AND METHODS

Materials

Male Sprague Dawley rats (Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences), weighing 200-300 g, were used in all experiments.

Thapsigargin was obtained from Alexis Company. 2-aminoethoxydiphenyl borate (2-APB), SK&F96365 were obtained from Merck KcaA (Darmstadt, Germany). Collagenase IV, HEPES, EGTA, trypan blue, econazole, miconazole, CsCl, CsOH and the other chemicals were from Sigma (USA). The pipette solution contained 120.0 mmol/L CsCl, 1.0 mmol/L MgCl₂, 10.0 mmol/L EGTA, 10.0 mmol/L HEPES, 2 μmol/L thapsigargin, pH 7.3 (adjusted with CsOH). The bath solution contained 145 mmol/L NaCl, 2.8 mmol/L KCl, 10.0 mmol/L CaCl₂, 1.0 mmol/L CsCl, 2.0 mmol/L MgCl₂, 10.0 mmol/L glucose, 10.0 mmol/L HEPES, pH 7.3 (adjusted with NaOH). Ca²⁺-Mg²⁺-free Krebs-ringer-HEPES buffer solution contained 5 mmol/L KCl, 1 mmol/L KH₂PO₄, 115 mmol/L NaCl, 25 mmol/L HEPES, 0.5 mmol/L EGTA, pH 7.3 (adjusted with NaOH).

Hepatic I/R injury model

The model of rat partial hepatic I/R injury was established according to the procedures reported by Colletti *et al.*^[5] with minor modifications. Briefly, adult animals were anesthetized with pentobarbital sodium (50 mg/kg) and heparinized (1 U/g) via an intraperitoneal injection. A midline laparotomy was performed, then an atraumatic clip was used to interrupt the arterial and portal venous blood supply to the cephalad three lobes of liver. After 20 min for hepatic ischemia, the clip was removed followed by 40 min for hepatic reperfusion. Sham-operated control animals were treated in an identical fashion with the omission of vascular occlusion.

Cell preparation

Rat kupffer cells were enzymatically isolated from SD rats. Briefly, after the model was achieved, the portal vein and inferior vena cava were cannulated. The liver was initially perfused at a flow rate of 20-30 mL/min with a constant-flow system with modified oxygenated Ca²⁺-Mg²⁺-free Krebs-ringer-HEPES buffer solution (37°C) for about 5 min, followed by Krebs-ringer-HEPES buffer solution (37°C) containing collagenase IV for 10 min. After the perfusions, cephalad three lobes of the liver were excised and minced in Ca²⁺-Mg²⁺-free Krebs-ringer-HEPES solution (37°C) for 20 min. The cells were filtered through a 200 μm nylon mesh, and washed by centrifugation at 700 g for 7 min. Then we reserved sediment and poured about 20-30 mL PBS into the sediment, washed it by centrifugation at 50 g for 4 min, reserved supernatant and washed it by centrifugation at 700 g for 5 min, plated the sediment on glass cover-slips and incubated it at 37°C in DMEM for 1-2 h. Then the cells were washed 3-5 times with the bath solution. The cells attached to the bottom of glass cover-slips were kupffer cells. The spherical, smooth cells were used for the whole-cell patch-clamp studies. All experiments were performed at room temperature (20-24°C).

Electrophysiological recordings

An automatic micropipette puller (Model P-97, Sutter Instruments, Novato, CA) was used to pull the electrodes. The resistance of the capillary glass electrodes (GG217,

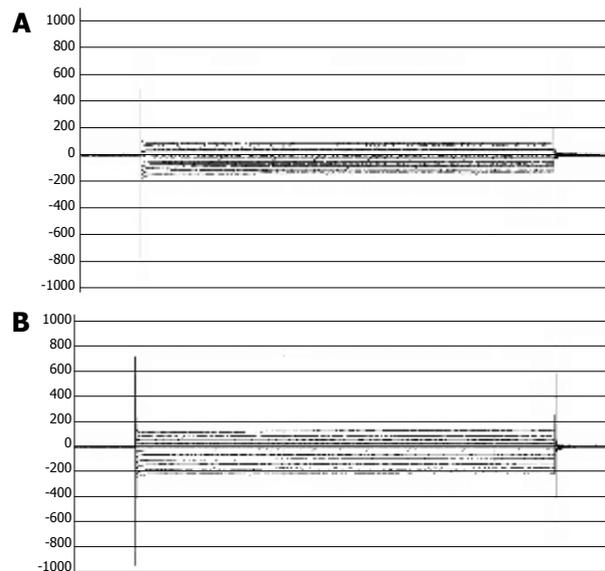


Figure 1 Whole-cell currents measured at the test potential from -120 mV to +80 mV in sham-operated control (A) and in rats with ischemia/reperfusion injuries (B).

World Precision, USA) used was 4-8 MΩ when filled with internal solution. An Axopatch 200B amplifier (Axon Instruments, USA) was used to record whole-cell currents with the filter set at 3 kHz, digitized at 5 kHz. The protocol of a series of depolarizing pulses from a holding potential of 0 mV to different membrane potentials (-120 mV to +80 mV) with a 20 mV increment was used for voltage clamp. Data analysis was performed using software (pCLAMP9.0, Axon Instruments, USA). The data were stored in computer for subsequent analysis. The blockers were added to the place around the cells with a rapid solution changer to investigate their effects on Isoc.

Statistical analysis

All values were expressed as mean ± SD. Appropriate *t*-test was used for the statistical analysis. ^a*P* < 0.05 and ^b*P* < 0.01 were considered statistically significant and apparently significant respectively.

RESULTS

Viability of isolated rat Kupffer cells

The isolated Kupffer cells showed polymorphism with typical polygon-like and star-like shapes. The purity and adhesion rate were 85% and 39.4%, respectively. The viability was over 80%. The isolated rat Kupffer cells were suitable for whole-cell patch clamp technique.

Isoc in isolated rat Kupffer cells

After the whole-cell configuration was established, cell membrane potential was clamped at 0 mV, and different test potentials from -120 to +80 mV with a 20 mV increment at a frequency of 0.2 Hz were applied. Following break-in, the currents in ischemia/reperfusion group (-159.5 ± 34.5 pA, *n* = 30) were greater than those in sham-operated control group (-80.4 ± 25.2 pA, *n* = 30) at the test potential of -100 mV (^b*P* < 0.01, Figure 1).

Table 1 Effects of 2-APB on Isoc at the test potential of -100 mV (mean \pm SD, $n = 8$)

Group ($\mu\text{mol/L}$)	Peak current (pA)	τ	Inhibitive rate (%)
Control	-227.8 \pm 68.5		
2-APB (20)	-139.6 \pm 52.9 ^b	7.6	39.0 \pm 10.3
2-APB (40)	-109.6 \pm 36.4 ^b	6.2	50.8 \pm 12.2
2-APB (60)	-88.1 \pm 37.6 ^b	6.7	61.0 \pm 15.1
2-APB (80)	-72.4 \pm 37.1 ^b	6.8	67.7 \pm 15.1
2-APB (100)	-57.8 \pm 33.4 ^b	6.9	73.7 \pm 14.3

^b $P < 0.01$ vs control.**Table 2** Effects of SK&F96365 on Isoc at the test potential of -100 mV (mean \pm SD, $n = 11$)

Group ($\mu\text{mol/L}$)	Peak current (pA)	τ	Inhibitive rate (%)
Control	-161.7 \pm 58.7		
SK&F96365 (5)	-82.6 \pm 29.5 ^b	5.3	46.1 \pm 17.3
SK&F96365 (10)	-57.0 \pm 24.9 ^b	6.1	61.2 \pm 20.5
SK&F96365 (20)	-47.1 \pm 20.5 ^b	6.5	67.2 \pm 19.5
SK&F96365 (40)	-35.6 \pm 15.4 ^b	7.1	74.8 \pm 15.2
SK&F96365 (50)	-25.3 \pm 11.9 ^b	7.7	82.1 \pm 11.0

^b $P < 0.01$ vs control.

But the I-V curve relation and reversal potential were not transformed (Figure 2).

Effects of 2-APB on Isoc

In order to observe the effects of 2-APB on Isoc, 2-APB with different concentrations (20, 40, 60, 80, 100 $\mu\text{mol/L}$) was respectively added to periphery of Kupffer cells after the whole cell configuration was established. According to the whole-cell currents at the test potential of -100mV (Table 1), Isoc was blocked by 2-APB in a concentration-dependent fashion with the IC₅₀ of 37.41 $\mu\text{mol/L}$. But the I-V curve relation and reversal potential were not transformed (Figure 3A).

Effects of SK&F96365 on Isoc

In order to observe the effects of SK&F96365 on Isoc, SK&F96365 with different concentrations (5, 10, 20, 40, 50 $\mu\text{mol/L}$) was respectively added to periphery of Kupffer cells after the whole-cell configuration was established. According to the whole-cell currents at the test potential of -100mV (Table 2), Isoc was blocked by SK&F96365 in a concentration-dependent fashion with the IC₅₀ of 5.89 $\mu\text{mol/L}$. But the I-V curve relation and reversal potential were not transformed (Figure 3B).

Effects of econazole on Isoc

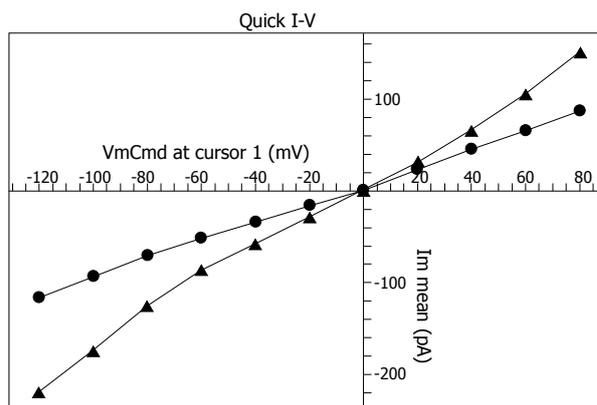
In order to observe the effects of econazole on Isoc, econazole with different concentrations (0.1, 0.3, 1, 3, 10 $\mu\text{mol/L}$) was respectively added to periphery of Kupffer cells after the whole-cell configuration was established. According to the whole-cell currents at the test potential of -100 mV (Table 3), Isoc was blocked by econazole in a concentration-dependent fashion with the IC₅₀ of 0.21 $\mu\text{mol/L}$. But the I-V curve relation and reversal potential were not transformed (Figure 3C).

Table 3 Effects of econazole on Isoc at the test potential of -100 mV (mean \pm SD, $n = 13$)

Group ($\mu\text{mol/L}$)	Peak current (pA)	τ	Inhibitive rate (%)
Control	-147.2 \pm 35.7		
econazole (0.1)	-83.5 \pm 22.8 ^b	6.7	42.0 \pm 15.1
econazole (0.3)	-64.7 \pm 18.9 ^b	8.7	54.6 \pm 14.7
econazole (1)	-56.6 \pm 19.9 ^b	9.8	60.7 \pm 13.7
econazole (3)	-47.9 \pm 18.2 ^b	10.3	66.5 \pm 13.5
econazole (10)	-39.9 \pm 15.9 ^b	11.1	71.9 \pm 12.6

^b $P < 0.01$ vs control.**Table 4** Effects of miconazole on Isoc at the test potential of -100 mV (mean \pm SD, $n = 10$)

Group ($\mu\text{mol/L}$)	Peak current (pA)	τ	Inhibitive rate (%)
Control	-133.2 \pm 32.0		
miconazole (0.1)	-86.4 \pm 31.7 ^b	7.1	35.6 \pm 14.9
miconazole (0.3)	-63.8 \pm 23.4 ^b	10.7	52.3 \pm 10.0
miconazole (1)	-45.9 \pm 13.1 ^b	9.2	64.5 \pm 10.6
miconazole (3)	-36.8 \pm 11.4 ^b	9.9	71.3 \pm 10.2
miconazole (10)	-29.7 \pm 10.5 ^b	9.9	76.5 \pm 9.4

^b $P < 0.01$ vs control.**Figure 2** I-V curve of whole-cell currents measured at -100 mV test potential. ^b $P < 0.01$ vs sham-operated control (●) (▲/R).

Effects of miconazole on Isoc

In order to observe the effects of miconazole on Isoc, miconazole with different concentrations (0.1, 0.3, 1, 3, 10 $\mu\text{mol/L}$) was respectively added to periphery of Kupffer cells after the whole-cell configuration was established. According to the whole-cell currents at the test potential of -100 mV (Table 4), Isoc was blocked by miconazole in a concentration-dependent fashion with the IC₅₀ of 0.28 $\mu\text{mol/L}$. But the I-V curve relation and reversal potential were not transformed (Figure 3D).

DISCUSSION

In this study, a rat hepatic I/R injury model was established. Kupffer cells were isolated and Isoc in Kupffer cells were detected.

Isoc can be activated by two ways, one is active way

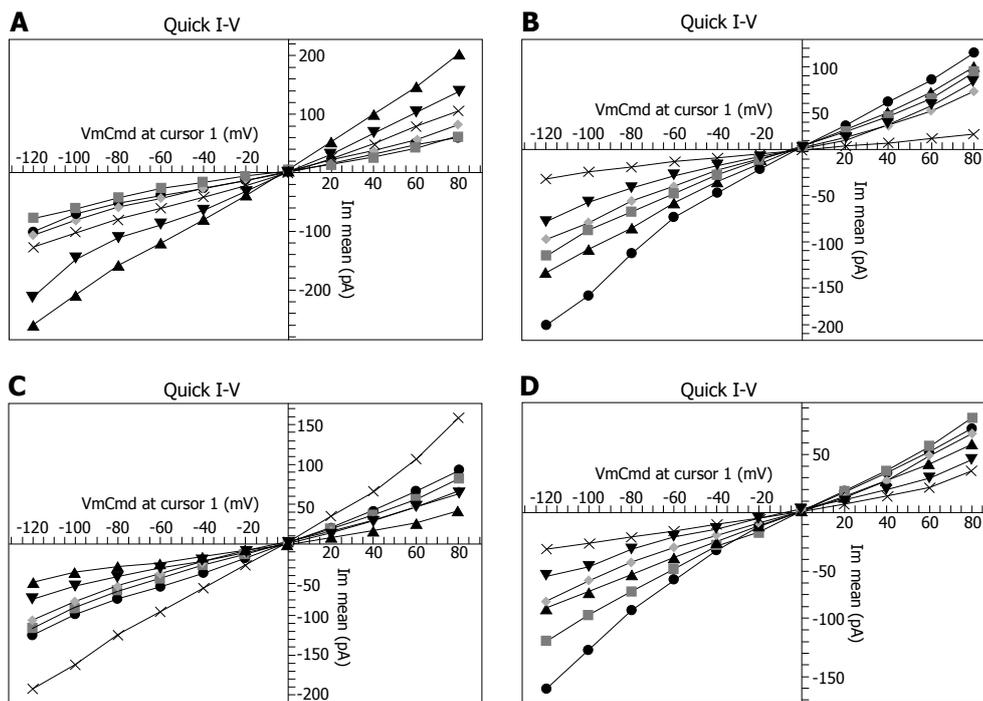


Figure 3 I-V curve of whole-cell currents at -100 mV test potential affected by 2-APB (A) (▲/I/R, ▼20 μmol/L, ×40 μmol/L, ●60 μmol/L, ■100 μmol/L), SK&F96365 (B) (●/I/R, ▲5 μmol/L, ×10 μmol/L, ▼20 μmol/L, ▼40 μmol/L, ×50 μmol/L), econazole (C) and (× I/R, ●0.1 μmol/L, ■0.3 μmol/L, ◆1 μmol/L, ▼3 μmol/L, ▲10 μmol/L), miconazole (D) in a concentration-dependent inhibiting fashion (●/I/R, ■0.1 μmol/L, ▲0.3 μmol/L, ◆1 μmol/L, ▼3 μmol/L, ×10 μmol/L).

induced by IP₃^[6-8], and the other way is EGTA induced by thapsigargin^[9]. The latter was chosen in our study.

Hepatic I/R injury can cause calcium overload in liver cells^[10-12]. Kupffer cells are considered to play a major role in hepatic I/R injury. The hepatic partial I/R process leads to activation of kupffer cells in ischemic and nonischemic areas of the liver, superoxide generation and proinflammatory cytokine production in Kupffer cells^[13]. An excess of reactive oxygen species (ROS) is generated by Kupffer cells activated during hepatic I/R injuries. All these events cause pathophysiological changes initiating a cascade of hepatocellular injury, necrosis, apoptosis, and subsequent inflammation^[14-16]. Inhibiting activation of Kupffer cells can relieve hepatic I/R injuries. Calcium overload is one of the important reasons for activation of Kupffer cells. In our study, I/R injuries could significantly increase Isoc of the Kupffer cells, suggesting that SOC has a close relation with hepatic I/R injuries and is the main channel for the influx of Ca²⁺ during calcium overload. *Broad et al*^[17] showed that phospholipase C and polyphosphoinositides can activate capacitative calcium entry (CCE). Recent findings indicate that receptor-mediated activation of phospholipase C in intact cells activates TRPC3 diacylglycerol production, independently of G proteins, protein kinase C, or inositol 1, 4, 5-trisphosphate^[18]. The last source of ion channel is the transient receptor potential (TPR) channel family, which forms non-selective cations^[19] all these indicate that hepatic I/R injuries activate phospholipase C which induces activation of store-operated calcium channels or TRPC3 channels, finally leading to calcium overload of Kupffer cells which could exacerbate hepatic I/R injuries.

Sequentially, Isoc of Kupffer cells after hepatic I/R injuries could be blocked by 2-APB, SK&F96365, econazole and miconazole in a concentration-dependent fashion.

2-APB has the restraint effect on Isoc in many kinds of cells^[20,21] and is a blocker of SOC and TRP channels^[22-24]. Our data have shown that Isoc of Kupffer cells after hepatic I/R injuries is blocked by 2-APB in a concentration-dependent fashion from 20 μmol/L to 100 μmol/L. 2-APB inhibits SOC by IP₃ way^[25,26]. Recent studies indicate that 2-APB may act as a direct blocker rather than as an IP₃ receptor antagonist while Icrac activity is rapidly blocked by extracellular 2-APB, but not by intracellular 2-APB^[27-30]. Furthermore, *Broad et al*^[17] showed 2-APB abolishes CCE induced by thapsigargin even in DT40 cells deficient for all isoforms of IP₃ receptor, consistent with a direct action of 2-APB on the SOC themselves. Our data also suggest that 2-APB may act as a direct blocker for SOC. Therefore, 2-APB protects Kupffer cells against being activated after hepatic I/R injuries by blocking Isoc.

SK&F96365 could block Isoc in many cells such as HL-60 cells, thyroid gland FRTL-5 cells, thrombocytes as well as voltage-dependent Ca²⁺ channels (VDCCs) in GH3 pituitary cells and smooth muscle cells^[31], suggesting that SK&F96365 can be used as a blocker of SOC in non-excited cells without VDCCs. VDCCs have been proved to be Kupffer cells, but Ca²⁺ current induced by EGTA and thapsigargin could not be blocked by verapamil (a kind of blocker of VDCCs). In our study, Isoc of Kupffer cells after hepatic I/R injuries was blocked by SK&F96365 in a concentration-dependent fashion. The mechanism is not completely clear. Since SK&F96365 could block two different channels, SOC and VDCCs, it may act on the SOC directly.

Econazole and miconazole decrease intracellular calcium levels after activation of SOC channels^[32] and block Isoc in Jurkat T-cells, HL-60 leukocytes, HEL cells, *etc.* Our data demonstrate that Isoc of Kupffer cells after hepatic I/R injuries is blocked by econazole and miconazole in a

concentration-dependent fashion.

In conclusion, hepatic I/R injuries can activate Kupffer cells, probably by increasing Isoc in Kupffer cells and activated Kupffer cells exacerbate hepatic I/R injuries. 2-APB, SK&F96365, econazole and miconazole can inhibit Isoc of Kupffer cells after hepatic I/R injuries in a concentration-dependent fashion. They have obvious protective effects on I/R injury, probably by inhibiting Isoc of Kupffer cells and preventing activation of Kupffer cells.

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Colorectal mucosal histamine release by mucosa oxygenation in comparison with other established clinical tests in patients with gastrointestinally mediated allergy

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Abstract

AIM: This study evaluated colorectal mucosal histamine release in response to blinded food challenge-positive and -negative food antigens as a new diagnostic procedure.

METHODS: 19 patients suffering from gastrointestinally mediated allergy confirmed by blinded oral provocation were investigated on grounds of their case history, skin prick tests, serum IgE detection and colorectal mucosal histamine release by *ex vivo* mucosa oxygenation. Intact tissue particles were incubated/stimulated in an oxygenated culture with different food antigens for 30 min. Specimens challenged with anti-human immunoglobulin E and without any stimulus served as positive and negative controls, respectively. Mucosal histamine release (% of total biopsy histamine content) was considered successful (positive), when the rate of histamine release from biopsies in response to antigens reached more than twice that of the spontaneous release. Histamine measurement was performed by radioimmunoassay.

RESULTS: The median (range) of spontaneous histamine release from colorectal mucosa was found to be 3.2 (0.1%-25.8%) of the total biopsy histamine content. Food antigens tolerated by oral provocation did not elicit mast cell degranulation 3.4 (0.4%-20.7%, $P = 0.4$), while anti-IgE and causative food allergens induced a significant histamine release of 5.4 (1.1%-25.6%, $P = 0.04$) and 8.1 (1.5%-57.9%, $P = 0.008$), respectively. 12 of 19 patients (63.1%) showed positive colorectal mucosal histamine release in accordance with the blinded oral challenge responding to the same antigen (s),

while the specificity of the functional histamine release to accurately recognise tolerated foodstuffs was found to be 78.6%. In comparison with the outcome of blinded food challenge tests, sensitivity and specificity of history (30.8% and 57.1%), skin tests (47.4% and 78.6%) or antigen-specific serum IgE determinations (57.9% and 50%) were found to be of lower diagnostic accuracy in gastrointestinally mediated allergy.

CONCLUSION: Functional testing of the reactivity of colorectal mucosa upon antigenic stimulation in patients with gastrointestinally mediated allergy is of higher diagnostic efficacy.

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Key words: Gut; Histamine release; Mucosa oxygenation; Food allergy diagnostics; Gastrointestinally mediated allergy

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INTRODUCTION

Gastrointestinal complaints after the ingestion of certain foodstuffs often pose diagnostic problems in various clinical situations such as food hypersensitivity, enzyme deficiencies, irritable bowel syndrome, inflammatory bowel disease, dyspepsia, eosinophilic gastroenteritis and several others. However, identification of immunologically mediated food hypersensitivity at the gastrointestinal level remains problematic, since skin tests and allergen specific serum IgE detection (e.g. RAST) may fail to show clear signs of food-specific sensitisation^[1-5] and do not necessarily indicate symptomatic food allergy. This is also valid for functional tests using blood cells (basophil histamine, or leucotriene release), lymphocyte transformation tests or measurement of mediators in blood or serum^[6-8]. Oral provocation, referred to as the 'gold standard' for food allergy diagnosis, is both time consuming and cost intensive, may put the patient at a

more or less severe risk, allows only one food to be tested per day, is often difficult to evaluate and is without doubt an unpopular and irritating procedure for the patient^[1,5,8].

Consequently, different methodical approaches have emerged for the improvement and acceleration of the cumbersome diagnostic way to identify patients with food allergy primarily involving the gastrointestinal tract. Already in 1942, 1984 and 1997, direct endoscopic observation has repeatedly been claimed to be of diagnostic value for recognition of food allergy when antigenic solutions were applied to the intestinal mucosa^[9-11]. However, direct endoscopic evaluation of allergen application and endoscopic or fluoroscopic balloon perfusion techniques harbour similar disadvantages to those experienced in oral provocation tests. All of these tests require special conditions with strict medical supervision because of the risk of allergic reactions *in vivo* during endoscopy or allergen perfusion^[10-12]. Only a few allergens can be tested during one examination and, in case of an endoscopic allergen injection, the endoscopic procedure is prolonged by at least 20 min, providing a higher risk of procedure-related complications, bacterial translocation and discomfort to the patient^[10-14]. Finally, the outcome of these endoscopic methods and the results obtained from double-blind, placebo-controlled food challenge procedures have never been directly compared on a scientific basis. Nevertheless, this is absolutely necessary for an appropriate evaluation in routine practice with respect to their diagnostic effectiveness^[1,5,8].

Another possibility to identify food allergy at the gastrointestinal tract was described in 1989. Baenkler *et al* used endoscopically taken samples from the duodenum to show antigen-induced histamine release *ex vivo*^[15,16]. Since many patients with suspected gastrointestinally mediated allergy (GMA) have to undergo endoscopic procedures for differential diagnostic reasons, this approach has the advantage that the principle demonstration of a food-induced mediator release can be performed outside of the patient, thus avoiding the risk of allergic reactions *in vivo*. In addition, several food antigens can be tested simultaneously without further burden to the patient^[15-17]. However, performance of functional histamine release experiments requires certain laboratory equipment and, initially, should be compared with the 'gold standard' for food allergy diagnostics before introducing this approach into clinical practice.

Similar to Baenkler's approach using duodenal mucosa, functional histamine release should also be expected from mucosa of other regions of the intestine^[15-17], provided that these tissue samples contain large enough numbers of (mucosal) mast cells. However, *ex vivo* histamine release from viable tissue samples of the lower gastrointestinal tract in response to nutritive antigens has not yet been studied together with the standardised *in vivo* oral challenge tests in order to provide a direct comparison of the two diagnostic methods. For this reason, this study investigated the rate of histamine release from colorectal mucosal samples in a group of patients with proven food allergy and compared the results of *ex vivo* mucosa oxygenation with the outcome of standardised blinded oral provocation tests.

Table 1 Patient data, allergens used for oral provocation, colorectal mucosal histamine release, skin prick test, serum IgE detection (RAST test), and patient's history

Pat. No.	Sex	Age	Allergen	Oral provocation	Colorectal HR	Skin test	RAST IgE	H
1WT	F	44	Cheese	+	+	-	+	?
			Rye bran	-	-	+	+	?
2DF	F	49	House dust	+	+	+	+	?
			Rye pollen	-	+	-	+	?
3OL	M	41	Soya flour	+	+	+	+	+
			Rye flour	-	-	-	+	?
4CG	M	23	Rye flour	+	+	-	-	-
5NA	F	42	Wheat flour	+	-	+	+	?
			Soya flour	-	-	-	+	?
6IL	F	43	Wheat bran	+	+	-	+	?
			Barley flour	-	-	+	-	?
7GG	F	19	Soya flour	+	+	+	+	?
			Wheat flour	-	+	-	+	?
8FJ	F	25	Soya lecithin	+	-	+	+	+
			Milk	-	-	-	+	+
9MR	F		Milk	+	-	-	-	-
			Potato	-	-	-	-	-
10MB	F	23	Wheat/rye	+	-	+	+	-
			Egg	-	-	-	-	+
11FB	F	51	Wheat flour	+	+	-	+	+
12RA	M	38	Spices	+	+	-	-	+
			Milk	-	-	-	-	+
13KF	M	32	Apple	+	+	+	+	-
			Potato	-	-	-	-	-
14AC	F	40	Nuts	+	+	-	-	-
			Maize	-	-	-	+	-
15KF	M	38	Nuts	+	-	+	+	-
16TM	F	28	Nuts	+	-	-	-	-
17SJ	F	37	Nuts	+	-	-	-	-
18LS	M	44	Nuts	+	+	-	-	-
			Rice	-	-	-	-	-
19SL	M	29	Wheat flour	+	+	+	-	?
			Potato	-	+	+	-	?

HR: histamine release; no.: number; pat.: patient; H: patients' history with regard to causative allergens and tolerated foodstuff.

MATERIALS AND METHODS

Patients

A total of 19 patients (7 male, 12 female; median age 38.0, range 19-51 years) were included in this study (Tables 1 and 2). All patients gave their informed consent and the study protocol was approved by the local ethics committee (No. 331). All patients (100%) reported abdominal symptoms, nausea, pain, vomiting and/or diarrhoea (98%) after certain meals, while postprandial extraintestinal signs of allergy such as skin reactions, asthma bronchiale and allergic rhinoconjunctivitis occurred only in a small percentage of patients (32%). Every patient was assessed on grounds of their history and detailed skin prick tests of environmental allergens (moulds, fibres, bacteria, pollen, dust) and food allergens (fish, fruit, vegetables, grains and different types of wholemeal, flour, bran, tea, coffee, eggs, milk, and cheese). Serum IgE detection of the putative allergens was performed according to the patients' history or skin tests (Tables 1 and 2). In the case of uncertainties about non-tolerated foods, tests were conducted for basic foodstuff. Case history, skin test reactions and RAST results were then compared with the outcome of oral

Table 2 Clinical symptoms induced by blinded, placebo-controlled food challenge, atopy status and predominant type of allergy according to Coombs and Gell

Pat. No.	Main symptoms	Atopy status	Type of allergy
1	Diarrhoea, flush, pruritus	-	Type I (systemic IgE)
2	Abdominal pain, loose stools bloating	+	Type I (systemic IgE)
3	Vomiting, diarrhoea	+	Type I (systemic IgE)
4	Abdominal pain, urticaria	-	Type I (local IgE)
5	Diarrhoea, abdominal pain dyspepsia, vomiting	+	Type I (systemic IgE)
6	Diarrhoea	-	Type I (systemic IgE)
7	Vomiting, loose stools right lower quadrant pain	+	Type I (systemic IgE)
8	Profuse watery diarrhoea	-	Type I (systemic IgE)
9	Diarrhoea, bloating, tachycardia	+	Type III (immune complexes present) or IV (?)
10	Pruritus, Rhinitis, tachycardia bloating, diarrhoea	-	Type I (systemic IgE)
11	Colitis, diarrhoea, arthragia rhinitis	-	Type I (systemic IgE)
12	Bloody diarrhoea, hypotension, abdominal pain, bloating	-	Type I (local IgE) and /or type III (immune complexes present)
13	Fever, diarrhoea, hypotension	+	Type I (systemic IgE)
14	Diarrhoea, vomiting, abdominal pain	-	Type IV (cellular hyper-sensitivity ?)
15	Bloating, diarrhoea, eosinophilia	+	Type I (systemic IgE)
16	Atopic eczema, diarrhoea, colitis, abdominal pain	+	Type I (local IgE) and /or type IV (cellular hyper-sensitivity ?)
17	Rhinitis, vomiting, diarrhoea	+	Type I (local IgE)
18	Diarrhoea, bloating	+	Type I (local IgE)
19	Eosinophilia, bloating, diarrhoea	+	Type I (systemic IgE)

Atopy status was defined as positive, when history or clinical manifestation of the patient gave evidence for milk crust, atopic eczema, asthma bronchiale and/or allergic rhino-conjunctivitis. For definition of the allergy type, the most dominant immunological signs were chosen to classify the ongoing allergic mechanisms in this population of patients with manifest gastrointestinally mediated allergy. However, some patients displayed symptoms that suggested more than one definitive type of allergy (see for example patient No. 9, 12, 16): Type I allergy (systemic IgE) was recognised when positive skin or antigen specific IgE levels were present in serum, type I allergy (local IgE) was diagnosed when intestinal lavage fluid contained elevated levels of IgE^[20]. Type III allergy was found in patients no. 9 and 12 who showed formation of either IgA, IgM and/or IgE immune complexes during or after allergen application by blinded food challenge. Additionally, type IV allergy was suspected in patients with heightened serum TNF levels during food challenge, and additionally in patient no. 14 who had a positive antigen-specific lymphocyte proliferation test.

challenge tests.

Food allergy was confirmed in each patient by blinded, placebo-controlled food challenge tests (BPCFC) adding the putative allergen to a basic diet containing rice, potato, oligopeptides (Survimed OPD, Fresenius, Germany) and tea. For provocation of flours (wheat, rye, soya, barley), commercially available allergen solutions for skin tests were used (Maser, Bochum, Germany). These were applied orally, while all other allergens were freshly prepared and given to the patients via a nasogastric tube^[5,18].

BPCFC was performed in a standardised fashion, while patients were hospitalised. Food antigen was administered in three different doses. Initially, a 1/20 dilution of the native allergen solution was administered, followed by 1/10 of the dose 3 h later and finally, a dose of 5 mL of the full strength native allergen solution was

provided^[18,19]. One single food antigen was tested per day. Placebo consisted of an oligopeptide-diet (protein source: hydrolysed soybean, Survimed OPD, Germany), which was also used for base-line nutrition (minimum: 1800 kcal/d), in conjunction with a rice-potato diet in order to prevent a catabolic state^[18,19]. A single blind challenge was performed in 42% (patients unaware of provocation protocol), while a double-blind challenge was carried out in 58% (patients and physicians unaware of the provocation protocol)^[18,19]. Blinding of the food antigens was managed by nutritionists, who were responsible for the preparation and addition of the allergens to usually tolerated foodstuff or to the oligopeptide solution, respectively.

Physicians selected the type of food to be tested either on the basis of the patients' history, previous results of skin prick tests and RAST tests or from a list of basic foodstuff. During the provocation procedure, the patients were provided with a peripheral venous line, and all medical staff involved was trained for medical intervention in case of an anaphylactic reaction. For the definition of food allergic reactions, a modified scoring system for symptoms was applied^[18] and main symptoms of patients evoked by the food challenge are listed in Table 2.

Food hypersensitivity was diagnosed only when food-specific immune events were detected through positive skin tests (mean wheal diameter of 3mm or greater than negative control^[1,9,18,19]), serum RAST-IgE (\geq class II^[8,19]) or through proof of intestinal IgE by endoscopically guided segmental lavage^[18,19] in conjunction with a reproducible clinical adverse reaction to the food antigen(s) applied^[1,5,8,18,19]. During BPCFC, at least one reproduction of an allergen induced clinical reaction and one or two placebo challenges were included for every patient. Whenever possible, both antigens causing clinical symptoms as well as tolerated antigens were applied to the patient, or else investigated on grounds of case history, skin tests, RAST and mucosal histamine release. In this way, a provocation allergen and a control allergen (Table 1) was determined for most patients (14 of 19 patients 74%), which enabled the direct comparison of the mucosal histamine release results with those of the BPCFC.

Before the execution of food challenge tests, additional examinations including endoscopy and histology of the upper and lower gastrointestinal tract were conducted^[5,18-20]. Patients with macroscopic alterations of the mucosa or with histological signs of acute inflammation (Crohn's Disease, ulcerative colitis *etc*) were excluded from the study as well as those suffering from other digestive diseases (e.g. celiac disease, autoimmunopathy, mastocytosis, eosinophilic gastroenteritis *etc*).

At least two weeks in advance of colonoscopy and BPCFC, any antiallergenic, immunosuppressive or steroid treatments had been discontinued for all patients. Patients were prepared for colonoscopy using a commercial polyethylenglycol solution. To facilitate colonoscopy, benzodiazepins (midazolam, diazepam) and meperidine were used at a dose of 2.5-10 mg (midazolam, diazepam) and 25-150 mg (meperidine), respectively^[20,21].

Colorectal mucosal histamine release

For colorectal mucosal histamine release by mucosa

oxygenation, 138 samples from the left-sided colon were obtained from all 19 patients. In 14 of 19 patients (74%), 10-12 mucosal samples were taken during colonoscopic examination. Whenever possible, 8 biopsies (4 repeats) were used for mucosa oxygenation and 2-4 for histological examination. The biopsies were immediately placed into a portable mucosa oxygenator (Intestino-Diagnostics, Erlangen, Germany) containing tubes filled with 2000 μL of oxygenated Hank's solution (pO_2 85-95 mmHg, pH 7.0, 37°C)^[16,17,20]. Each incubation medium was bubbled with a steady flow of room air to ensure sufficient oxygen pressure inside the biopsy, to facilitate allergen distribution into the tissue or mediator release from the tissue and to avoid ischemic damage of the tissue^[16,17,20,21].

Histamine release into the culture medium was measured at 0, 7.5, 15, and 30 min by removal of 200 μL of the supernatant at each sampling time^[17,20,21]. To obtain as accurate histamine measurements as possible, each 200 μL sample was immediately denatured by heating to 95°C for 5 min in order to destroy all histamine metabolising enzymes that may have been contained within the drawn supernatant^[16,17,20,21].

Allergen induced histamine release was achieved by addition of 200 μL Hank's solution containing 5 μL of native allergen solution to the culture medium at the sampling time of 0 min, thus providing a final concentration of 0.01 μg allergen/mL. The same procedure was applied for positive control using anti-human immunoglobulin E except that 20 μL of pure anti-IgE solution (Behringwerke, Marburg, Germany) were used for dilution. The final concentration of anti-human-IgE was 0.01 $\mu\text{g}/\text{mL}$, which has previously been found to be the optimal stimulation concentration^[16,17]. For negative control (spontaneous mucosal histamine release), only 200 μL Hank's solution were added to the culture medium. The stimulation procedure of the 8 samples of each patient was arranged as follows: two samples were studied for spontaneous mucosal histamine release (negative control), two for anti-IgE induced histamine release (positive control), two for a BPCFC-positive allergen (provocation allergen) and two for provocation negative, i.e. tolerated antigens (control allergen).

After a stimulation period of 30 min, the rest of the volume of 1400 μL containing the biopsy (1200 μL Hank's + 200 μL stimulus) was also heated to 95°C for five minutes in order to determine the remaining tissue histamine content and to denature tissue histamine catabolising enzymes^[17,20,21].

Histamine measurement

Histamine was measured using a sensitive and specific radioimmunoassay (Histamine RIA, Beckman-Coulter, Krefeld, Germany)^[17,21,22]. The actual rate of histamine release was expressed as the percentage of the total tissue histamine content of the biopsy. This was calculated from the discharged histamine into the supernatant and the remaining histamine content in the tissue at the sampling time of 30 min^[17,20-22]. Intra-assay and inter-assay coefficients of variation for the histamine radioimmunoassay ($n > 150$ samples) were 6.2% and 8.8% for supernatants, and 13.7% and 18.2% for detection of

Table 3 Results from colorectal mucosal histamine release (% of total tissue histamine content) following *ex vivo* mucosa oxygenation in patients with GMA

Pat. No.	Spontaneous HR	Anti-IgE	Control Allergen	Provocation allergen
1	1.7	5.4	3.1	8.7
2	2.6	12.6	11.5	17.3
3	3.4	5.2	4.5	39.1
4	3.2	6.2	-	8.8
5	3.3	6.0	3.1	3.0
6	0.5	3.7	0.9	5.1
7	4.1	1.6	8.9	27.6
8	7.5	2.9	8.3	3.2
9	7.0	6.6	4.9	8.1
10	2.5	3.6	3.0	4.8
11	0.3	3.6	-	1.5
12	1.4	7.9	2.4	11.3
13	5.3	-	3.7	11.2
14	22.4	25.6	20.7	57.9
15	25.8	23.0	-	28.7
16	1.5	-	-	1.8
17	4.8	9.3	-	6.7
18	2.9	3.8	2.8	5.8
19	0.1	1.1	0.4	2.4
Median:	3.20	5.40 ^b	3.40	8.10 ^a
(range)	0.1-25.8	1.1-25.6	0.4-20.7	1.5-57.9

Significance levels: ^a $P = 0.008$ vs spontaneous histamine release; ^b $P = 0.04$ vs spontaneous histamine release. HR: histamine release (average value from two measurements); no.: number; pat.: patient; anti-IgE: anti-immunoglobulin E. Control and provocation allergen are allergens, which were either tolerated or induced clinical symptoms during blinded oral food challenge procedures. Rates of histamine release are expressed as percentage (%) of total tissue histamine content. Each release value represents the average value from two repeat experiments (two separate biopsies).

the remaining tissue histamine content, respectively. The individual rates of histamine release were found to vary by up to 24.0% within the same person.

Histamine content was also measured in native allergen solutions to exclude histamine contamination.

Statistical analysis

From each patient, two rates of histamine release were obtained for each of the parameters spontaneous histamine release, anti-IgE, provocation and control allergen. The average value for each pair of release data was calculated and used for final statistics as listed in Table 3.

For descriptive statistics of the whole group, the median and range were chosen. Statistical comparisons were made using the *U*-test (Wilcoxon, Mann & Whitney) and significance levels are given in brackets. For comparison of the clinical tests, *ex vivo* biopsy stimulation by mucosa oxygenation was considered successful (positive) when the antigen containing solution caused an increase of the histamine release up to more than twice that of the spontaneous release.

RESULTS

Histamine release from colorectal tissue

Spontaneous histamine release from viable colorectal mucosal fragments amounted to only 3.2% of the total tissue histamine content, indicating that mast cells are able

Table 4 Colorectal mucosal histamine release in comparison with the outcome of oral provocation tests in patients with GMA

	Positive HR	Negative HR	Line sum
Positive BPCFC	12	7	19
Negative BPCFC	3	11	14
Column sum	15	18	

HR: histamine release, BPCFC: (double- or single) blinded, placebo-controlled oral food challenge. Colorectal mucosal histamine release: Sensitivity 63.1%, specificity 78.6%.

Table 5 Patients' history in comparison with the outcome of oral provocation tests in patients with GMA

	Positive H	Negative H	Line sum
Positive BPCFC	4	9	13
Negative BPCFC	3	4	7
Column sum	7	13	

H: patients' history, BPCFC: (double- or single) blinded, placebo-controlled oral food challenge. Note: Only 13 of 19 patients (68%) felt confident to answer questions concerning suspected provocation antigens, while the remaining were uncertain or had no experience of adverse reactions to the particular antigen. For evaluation of the specificity of patients' history, data from only 7 patients (37%) were available. Patients' history: Sensitivity 30.8%, specificity 57.1%.

to maintain their normal metabolism and their mediators within granules during mucosa oxygenation (Table 3). Application of anti-human-IgE induced a clearly enhanced rate of histamine release of 5.4% ($P = 0.04$) within 30 min, confirming the functional reactivity of mucosal immune effector-and intestinal mucosal mast cells towards IgE-receptor cross-linking^[15-17,21,23]. Four of 17 patients (23.5%) were found to be unresponsive to the anti-IgE concentrations used. Interestingly, these patients had the highest rates of spontaneous histamine release, possibly indicating that mast cells had already been degranulated or that a high rate of spontaneous histamine secretion may exert some negative feed-back mechanisms on mast cell triggering by anti-IgE (Table 3).

Incubation of intact colorectal tissue with BPCFC-negative food antigens did not induce a significant increase in histamine release (median increase 1.2 fold; range 0.6-4.4 fold of spontaneous mediator release) in patients with GMA. Histamine release with control antigens amounted to 3.4% and was not statistically different from spontaneous histamine release (Table 3).

In contrast, provocation allergens that evoked clinically significant reactions in allergic individuals (BPCFC-positive food antigens) already induced a 2.6 fold increased rate of histamine release compared to the spontaneous release (range 0.9-24 fold) during 30 min of mucosa oxygenation. The percentage of histamine release in response to provocation allergens was 8.1% and significantly different from spontaneous histamine release ($P = 0.008$).

Colorectal mucosal histamine release in comparison with established clinical tests

Colorectal mucosal histamine release was positive in 12 of

Table 6 Skin prick tests in comparison with the outcome of oral provocation tests in patients with GMA

	Positive prick test	Negative prick test	Line sum
Positive BPCFC	9	10	19
Negative BPCFC	3	11	14
Column sum	12	21	

BPCFC: (double- or single) blinded, placebo-controlled oral food challenge
Skin prick test: Sensitivity 47.4%; specificity 78.6%.

Table 7 Allergen-specific serum IgE determinations (RAST test) in comparison with the outcome of oral provocation tests in patients with GMA

	Positive RAST	Negative RAST	Line sum
Positive BPCFC	11	8	19
Negative BPCFC	7	7	14
Column sum	18	15	

BPCFC: (double- or single) blinded, placebo-controlled oral food challenge
Allergen-specific IgE detection in serum: Sensitivity 57.9%; specificity 50.0%.

19 patients (sensitivity 63.1%, Table 4), who experienced a reproducible clinical reaction in response to the same provocation antigen. In contrast, 3 of 14 patients (21.4% false positive) discharged significant histamine amounts although oral provocation was negative. Control antigens, tolerated by the patient during BPCFC, were also found to be negative with regard to histamine release in 11 of 14 patients (specificity 78.6%).

When comparing established clinical parameters for food allergy diagnostics with the outcome from blinded provocation tests (gold standard), a lower diagnostic accuracy was obtained through reference to patients' history (Table 5), skin test results (Table 6) and allergen-specific IgE detection in serum (Table 7) than with histamine release experiments.

The comparison between patients' history and BPCFC (Table 5) was somewhat impeded, since only 13 of 19 patients (68.4%) knew their allergen inducing clinical symptoms, 7 of 14 patients (50%) with recurrent gastrointestinal complaints tried to give exact answers on questions about their well tolerated foods, while all other individuals had significant uncertainties about adverse reactions to or tolerance of the food antigens tested.

DISCUSSION

Diagnosis and existence of gastrointestinal food allergy are still a matter of debate^[1,4,5,18]. To date, no exact diagnostic and practical relevant means are readily available for the gastroenterologist or endoscopist to examine the gastrointestinal mucosa for signs of food hypersensitivity, when patients with recurrent episodes of variable gastrointestinal complaints are referred for further diagnostics^[5,8,10]. One innovative approach for the diagnosis of food hypersensitivity by endoscopy may be the use of a mucosa oxygenation system, which allows culturing of small viable endoscopic samples outside of

the patient for immunological release experiments^[15-17,20,23]. Since histamine is one important and very early secreted mediator of different types of IgE and non-IgE mediated allergic reactions^[1,3,17,24], this study was designed to evaluate colorectal mucosal histamine release from patients with gastrointestinal food hypersensitivity. In contrast to allergic individuals with typical extraintestinal signs of type I food hypersensitivity, diagnostic problems have been repeatedly reported in this patient population primarily involving the gastrointestinal tract with symptoms of allergic diarrhoea, vomiting, nausea, *etc.* In addition, the frequency of local gastrointestinal allergy in several important clinical conditions is also still unclear^[1,4,5,8-11,25-27].

This study demonstrates that a significant histamine release can be induced from colorectal mucosa upon IgE receptor cross-linking or antigenic stimulation *ex vivo* by mucosa oxygenation using endoscopically taken samples^[15-17,23]. This confirms that functional antigen-specific tests using histamine as the primary test parameter are equally feasible with mucosa from the lower gastrointestinal tract as from the upper gastrointestinal tract (duodenum^[15]) or more long-lived mediators like mast cell tryptase or eosinophilic cationic protein^[20,23]. However, in contrast to previously published work featuring tryptase or eosinophilic cationic protein, histamine release tests using bioptic tissue bear the distinct practical advantage that they can be performed within a short period of time of only 30 min, providing the appropriate technique to rapidly destroy all histamine catabolising enzymes in the drawn culture supernatants is applied^[16,17,20,21]. The quick performance of mucosa oxygenation using histamine as a diagnostic parameter may qualify this test for its use in clinical practice, possibly as a refinement or complement of existing endoscopic-diagnostic procedures when patients with suspected gastrointestinal food allergy are being referred for diagnostic work-up.

Compared to other human tissues or isolated mast cells, histamine release from colorectal mucosa was found to be of a smaller magnitude than expected. This could be a result of the short cultivation period and of the fact that colorectal tissue harbours large quantities of histamine metabolising enzymes, which are still active within the viable cultured tissue at a physiological rate during mucosa oxygenation^[6,11,17,20,21,28]. However, compared with the spontaneous rate of histamine release, functional mast cell stimulation by anti-IgE or BPCFC-positive antigens achieved a significantly higher rate of histamine release, while control antigens showed a similar degree of histamine release as the spontaneous secretion. This study proved unambiguously the reactivity of histologically normal gut mucosa in allergic patients upon specific challenge. In view of that, gastroenterologists will find a valuable addition to their diagnostic methods through the establishment of a functional test using biopsies of the involved and reacting allergic shock organ^[15,21,23]. Depending on the number of biopsies taken during endoscopy, this functional test allows the simultaneous examination of different food antigens during mucosa oxygenation^[15-17,21]. In this way, any contact between the antigen and the patient's immune system is avoided and the patient is not put at risk of any allergic symptoms or

reactions.

Although only a small group of patients was investigated in this study, colorectal mucosal histamine release was found to yield a diagnostic sensitivity and specificity of 63.1% and 78.6%, while skin tests and serum IgE detection reached only a sensitivity and specificity of 47.3% and 78.6% or 57.5% and 50%, respectively. Similar low rates of diagnostic accuracy of the skin prick tests and RAST tests have also been reported by other investigators in GMA^[1,2,4,5,12,25-27]. This may be explained by the fact that sensitisation in the cutaneous and serological compartment may not always accurately reflect the immunological response of the mucosal microenvironment at the large surface area of the gastrointestinal tract^[11,18,25,27]. The presence of different (immunological) compartments with separate mechanisms for local IgE production in food hypersensitivity may also account for the known multitude of allergic manifestations in food allergy^[11,8,29]. This is illustrated by the fact that several individuals in this study experienced significant clinical symptoms during BPCFC despite negative skin or serum IgE tests, confirming the need for further diagnostic means directly targeting the involved shock organ^[1,5,25-27,29].

Mucosa oxygenation with histamine or more long-lived mediators bears the potential to predict the outcome of double-blind, placebo-controlled food challenges^[17,21,23]. Hence, a future cost-effective approach of the gastroenterologist to diagnose gastrointestinal food allergy could possibly be based on the additional use of gut mucosal histamine release (Table 4) to avoid time-consuming, cost-intensive and sometimes risky challenge procedures. However, the real value of such a diagnostic procedure has yet to be established within greater patient populations^[21,23]. The rationale of this proposal for future diagnostics is given by the fact that gut mucosal histamine release was proven to be of superior diagnostic value compared to that of the commonly used allergological means such as case history, skin prick tests or RAST tests, as these are not always concise or do not directly examine the involved organ in patients with GMA, respectively^[1,2,25,27]. The data presented here suggest that patients with a food antigen-induced mucosal histamine release exceeding more than twice the amount of the spontaneous one be could directly subjected to a specific elimination diet.

As part of appropriately provided health care, prospective long-term analysis of the clinical course of these patients is always necessary. Diminishing financial resources, however, dictate the need for more economical investigations, which may be sufficed by mucosa oxygenation. Although this approach is more invasive than blinded food challenges, it needs only one endoscopic examination with testing of several antigens, while DBPCFC needs at least one (in-patient) test day for each individual allergen application. The data presented here suggest that mucosa oxygenation could perhaps eliminate the need to perform DBPCFC in a significant number of patients suspected of having GMA^[16,17,21].

However, the fact that colorectal mucosal histamine release by mucosa oxygenation did not identify all BPCFC-positive allergens is more likely to be related to the

complex pathophysiology and compartmentalisation of gastrointestinal food allergy rather than to inadequately applied methods. Patients with positive provocation but negative colorectal histamine release, which was the case for 7 of 19 patients (36.8%), may develop their allergic reactions in more proximal parts of the gastrointestinal tract (e.g. stomach, duodenum). Or else, these patients may react only in blood or at extraintestinal sites, respond to the tested antigen mainly with other mediators rather than with histamine (e.g. arachidonic acid products, eosinophilic proteins *etc*) or may have produced the antigenic epitope after passage through the liver^[1,5,8,25,29]. Conversely, control allergens, which elicited a significant histamine release in 3 of 14 patients (21.4%), may fail to provoke a clinically significant reaction unless histamine catabolism is sufficiently active^[1,28]. In addition, the demonstration of food-specific histamine release from colorectal mucosa may also explain why some patients experience postprandial extraintestinal symptoms (urticaria, hypotension, asthma bronchiale *etc*) despite negative skin tests. In such cases, intestinally produced and released histamine may reach peripheral extraintestinal organs and activate their histamine receptors inducing classical extraintestinal allergic symptoms without the presence of food-specific IgE in the periphery. These different pathophysiological parameters in combination with several yet unknown or ill-defined factors (e.g. neurovegetative impulses, gut flora *etc*) contribute to or induce the great variability of clinical manifestations in GMA^[1,3-5,10,18,23,29].

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CLINICAL RESEARCH

Correlation of *p53* gene mutation and expression of P53 protein in cholangiocarcinoma

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Abstract

AIM: To characterize the tumor suppressor gene *p53* mutations and study the correlation of *p53* gene mutation and the expression of P53 protein in cholangiocarcinoma.

METHODS: A total of 36 unselected, frozen samples of cholangiocarcinoma were collected. *p53* gene status(exon 5-8) and P53 protein were examined by automated sequencing and immunohistochemical staining, combined with the clinical parameters of patients.

RESULTS: *p53* gene mutations were found in 22 of 36 (61.1%) patients. Nineteen of 36 (52.8%) patients were positive for P53 protein expression. There were significant differences in extent of differentiation and invasion between the positive and negative expression of P53 protein. However, there were no significant differences in pathologic parameters between the mutations and non-mutations.

CONCLUSION: The alterations of the *p53* gene evaluated by DNA sequence analysis is relatively accurate. Expression of P53 protein could not act as an independent index to estimate the prognosis of cholangiocarcinoma.

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Key words: Cholangiocarcinoma; *p53* gene; Mutation; DNA sequence

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INTRODUCTION

Tumor suppressor gene, besides oncogene, is involved in the development of cancer, which inhibits cell proliferation and formation of tumor. Normally tumor suppressor gene counteracts with oncogene to protect an organism against cancer. The *p53* tumor suppressor gene is the most common mutated gene in human cancer^[1-9], occurring in approximately 50% cancers^[10-12]. Cholangiocarcinoma is among the most common malignant tumors. Mutation of *p53* is one of the most frequently encountered genetic alterations in cholangiocarcinoma. *p53* mutations play a central role in carcinogenesis of cholangiocarcinoma.

Alterations of the *p53* gene can be evaluated by DNA sequencing analysis and immunohistochemistry(IHC). P53 protein overexpression is considered a poor prognosis of cholangiocarcinoma^[13-17]. But IHC has shown controversial results over that of DNA sequencing analysis. We examined mutation status of *p53* gene by automated sequencing and IHC in 36 cases of cholangiocarcinoma and studied the correlation of *p53* gene mutation with clinical parameters and its clinical significance.

MATERIALS AND METHODS

Patients

A total of 36 unselected, frozen samples were obtained from patients with cholangiocarcinoma who had been treated by surgical resection from April 2000 to May 2005 in the Department of General Surgery of the First Affiliated Hospital of China Medical University and Hepatobiliary Surgery of the Affiliated Yantai Yuhuangding Hospital of Qingdao University Medical College. The types of cholangiocarcinoma included 18 cases of tubular adenocarcinomas, 9 cases of papillary adenocarcinomas, 4 cases of mucoid carcinomas and 5 cases of undifferentiated carcinoma. Among them, well-moderately differentiated was 25 and poorly differentiated was 11 cases respectively. There were 16 cases of T1 stage, 10 cases of T2 stage and 10 cases of T3 stage by the UICC standard. Lymph node metastasis was seen in 33 cases. Non- lymph node metastasis was seen in 3 cases. The patients contained 23 males

Table 1 Primers sequences of p53 gene exons 5-8

Primers	Sequences	
	Sense	Anti-sense
Exon 5	5'-TGT TCA CTT GTG CCC TGA CT-3'	5'-CAG CCC TGT CGT CTC TCC AG-3'
Exon 6	5'-ACA GGG CTG GTT GCC CAG GGT-3'	5'-CTC CCAGAG ACC CCA GTT GC-3'
Exon 7	5'-GGT CTC CCC AAG GCG CAC TGG-3'	5'-AGG CTG GGG GCA CAG CAG GCC-3'
Exon 8	5'-ATT TCC TTA CTG CCT CTT GC-3'	5'-AAG TGA ATC TGA GGC ATA AC-3'

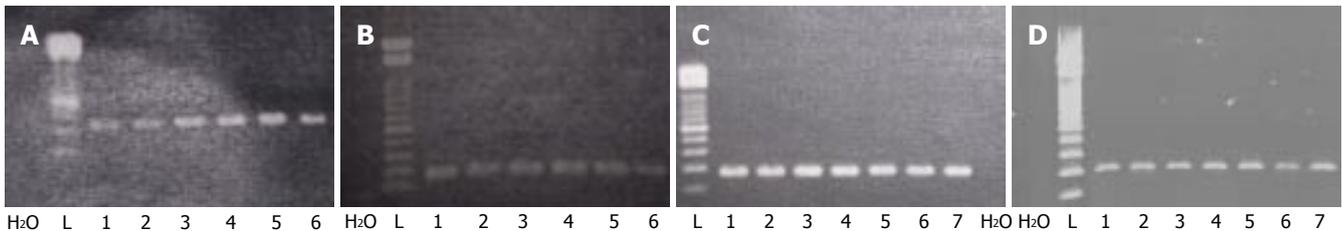


Figure 1 p53 exons 5, 6, 7, 8 PCR product. L : 100 bp DNA ladder; 1-7: Samples. A: Exons 5; B: Exons 6; C: Exons 7; D: Exons 8.

and 13 females, with age ranging from 36 to 71 (median, 61.2) years. All of the samples were frozen at -80°C until DNA extraction and subjected to histological diagnosis by a pathologist.

DNA extraction

DNA was extracted from tissues using a QIAamp DNA Micro kit: QIA (Germany). Tissue samples weighing less than 10 mg were placed into a 1.5 mL microcentrifuge tube. Immediately 180 μL buffer ATL and 20 μL proteinase K were added and mixed by pulse-vortexing for 15 s. Then they were incubated at 56°C overnight. Two hundred microliter buffer AL and 200 μL ethanol (100%) were added and incubated for 5 min at room temperature. After that, all of the lysates were applied onto the QIAamp MinElute column. Five hundred microliter buffer AW1 and buffer AW2 were added. After centrifugation, 100 μL buffer AE was applied to get DNA. DNA quantity was determined by the ratio of A260/280.

Primer sequences and PCR amplification

Table 1 shows primer sequences used for p53 exons 5-8, which was synthesized by Hokkaido Bioscience Co. (Japan). PCR used a 20 μL reaction volume containing 1 unit of Hot start EXTaq DNA polymerase (Takara, Biochemical, Japan), 2 μL of $10 \times$ EXTaq buffer, 2 μL of dNTP mixture and each primer (8 pmol each for reaction) and 1 μL of DNA template. The condition of the first PCR is as follows: 96°C for 3 min for denaturation, 40 cycles of 96°C for 30 s, 60°C for 30 s, 72°C for 30 s with a final elongation step of 4 min at 72°C . Water was used as a negative control. Five microliters of PCR product were analyzed on 1% TBE gel electrophoresis. Each sample was repeated three times.

DNA sequencing

All of the PCR products were purified using Auto seq TMG-50 (Amersham Biochemical Company, USA). Big-Dye Terminator Cycle sequencing Ready Reaction (Perkin Elmer, USA) was used. The primers of sequencing were

the same as PCR primers. But its concentration was one tenth of PCR primers. The condition is as follows: 95°C for 4 min, 95°C for 30 s, 55°C for 30 s, 72°C for 30 s for 40 cycles with a final step of 4 min at 72°C . Both sense and antisense chains were analyzed on an ABI prism 310 Genetic Analyzer (Perkin Elmer). Each sample was repeated three times.

Immunohistochemistry

Five-micron sections were dewaxed in xylene and rehydrated. Endogenous peroxidase was destroyed by a 15-min treatment in 30 mL/L hydrogen peroxide (H_2O_2) in phosphate-buffered saline (PBS) at room temperature. The sections were blocked with a combination of normal mouse serum and then incubated with anti-p53 protein (dilution 1:50, mouse anti-p53 protein by Boster Co.), followed by biotinylated-conjugated sheep anti-mouse IgG (Boster Co.). The complex was visualized by diaminobenzidine (Boster Co.). The specificity of the reaction was confirmed by use of negative control, blank control and substitution control, in which PBS substituted the secondary antibody biotinylated-conjugated sheep anti-mouse IgG.

Statistical analysis

The results were analyzed with χ^2 test. $P < 0.05$ was taken as significant.

RESULTS

p53 gene mutations and expression of P53 protein in cholangiocarcinoma tissues

Positive band of exons 5-8 was found in all samples after PCR amplification (Figures 1 A-D). p53 gene mutations were detected in 22 of 36 patients (61.1%) by DNA sequencing. Among them, there were 7 cases of exon 5 mutations, which were located on 161, 175 and 196 codons. All were transition (G:C/A:T). Six cases of exon 6 mutations were located on 209, 213 and 215 codons, of which 4 cases were of transition (G:C/A:T) and 2 cases

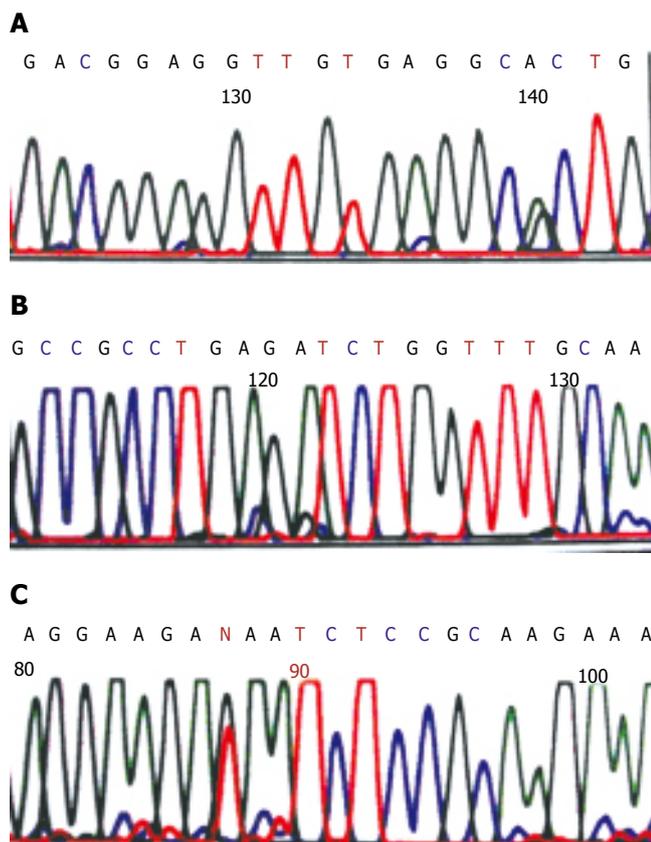


Figure 2 Tranversion of *p53* exons 5, 6, 8 in cholangiocarcinoma. **A:** Exons 5 (G:C/A:T); **B:** Exons 6 (G:C/A:T); **C:** Exons 8 (G→T).

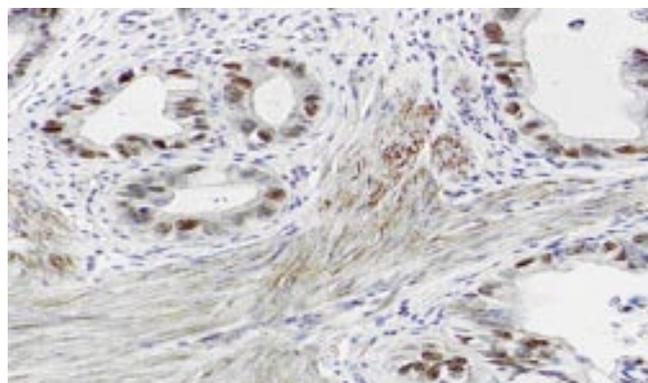


Figure 3 P53 protein was positive in cholangiocarcinoma (SABC × 400).

of tranversion (G-T). Three cases of exon 7 mutations were located on 248, 252 codons. Six cases of exon 8 mutations were located on 282, 287, 289, 306 codons, of which 4 cases were of transition (G:C/A:T) and 2 cases of tranversion (G-T) (Figures 2 A-C).

Nineteen cases (52.8%) were positive for P53 protein. P53 protein localized in the nuclei of cholangic epithelial cells. Moreover, it was crisp and finely granular (Figure 3).

Correlation of *p53* gene mutation and clinical parameters

There were significant differences in degree of differentiation and invasion between the positive and negative samples of P53 protein expression ($P < 0.05$, P

Table 2 Correlation of mutation status of *p53* gene and P53 protein expression with pathologic parameters in cholangiocarcinoma

Pathologic character	n	<i>p53</i> gene mutation		P53 protein expression	
		Positive (n, %)	P value	Positive (n, %)	P value
Pathologic type					
Tubular carcinoma	18	11 (61.1)		10 (55.6)	
Papillary carcinoma	9	6 (66.7)		5 (55.6)	
Mucoid carcinoma	4	2 (50.0)		2 (50.0)	
Non-differentiated carcinoma	5	3 (60.0)	> 0.05	2 (40.0)	> 0.05
Differentiation					
Well-Moderate	25	13 (52.0)		9 (36.0)	
Poor	11	9 (81.8)	> 0.05	10 (90.9)	< 0.05 ^a
Invasion					
T1	16	7 (43.8)		2 (12.5)	
T2	10	8 (80.0)		8 (80.0)	
T3	10	7 (70.0)	> 0.05	9 (90.0)	< 0.05 ^c
Lymph node metastasis					
N0	3	2 (66.7)		0 (0)	
N1	33	20 (60.6)	> 0.05	19 (57.5)	> 0.05

< 0.05). However, there were no significant differences in age, gender, degree of differentiation and invasion, lymph node metastasis, stage between the mutations and non-mutations ($P > 0.05$) (Table 2).

DISCUSSION

Cholangiocarcinoma is the second most common cancer of the hepatobiliary system. In recent years, the incidence and mortality of cholangiocarcinoma have been increasing in China^[18,19]. In most patients, the disease is only diagnosed at a late stage. Patients with obstructive jaundice are frequently at the advanced stage of the disease, which is contraindicated for operation^[20-26]. The development of molecular biology, the identification of molecular factors involved in cholangiocarcinoma carcinogenesis, and the elucidation of the mechanisms will significantly impact prevention, diagnosis, treatment and prognosis. *p53* gene is located on the short arm of chromosome 17, which consists of 11 exons. There are four mutation hot-spots(132-143, 174-179, 236-248, 272-281) within the core domain (exon 5-8), which are the key sites of biological activity of P53 protein^[27,28]. *p53* gene is an important regulator factor of cell proliferation. It is related to cell cycles, DNA repair, cell differentiation and apoptosis. In the presence of DNA damage the expression of *p53* is enhanced and induces G1 cell cycle arrest until DNA is repaired. If repair is insufficient, *p53* gene promotes apoptotic cell death. However, *p53* gene mutation cannot block cell proliferation. Through cooperation with inactivation of tumor suppressor genes and activation of oncogenes, cells transform into malignant ones and become a tumor. *p53* tumor suppressor gene is the most common mutated gene in human cancer and is frequently seen in cholangiocarcinoma. Recent studies have found that positive expression of P53 protein is related to invasion and lymph node metastasis in cholangiocarcinoma^[15,16].

p53 gene mutation could act as an index to estimate the prognosis of cholangiocarcinoma.

We examined mutation status of *p53* gene exons 5-8 by automated sequencing in 36 cases of cholangiocarcinoma. We found *p53* gene mutations in 22 of 36 (61.1 %) patients. Nineteen of 36 (52.8%) patients were positive for P53 protein expression. There were significant differences in extent of differentiation and invasion between positive and negative expression of P53 protein. However, there were no significant differences in age, gender, extent of differentiation, invasion, lymph node metastasis, and stage between the mutations and non-mutations.

Wild-type *p53* (non-mutated) has a short half-life of about 20 min. Mutant P53 protein has a greater stability with half-life prolonged up to 1.4-7 h. It can be detected by IHC method. But the use of different P53 antibodies and methods can result in a marked difference in the degree of overexpression, and varying levels of overexpression may also be noted in the same tumor specimen. IHC has been shown to have discordancy rates of 30%-35% compared with DNA sequencing. Thus, the determination of *p53* overexpression is not an accurate measure of *p53* function. Although DNA sequence analysis is a cumbersome, time-consuming and difficult method on archived material, it could provide a more accurate means of detecting *p53* mutations. Thus, we think *p53* gene mutation could not act as an independent index to estimate the prognosis of cholangiocarcinoma.

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S- Editor Pan BR L- Editor Zhu LH E- Editor Ma WH

RAPID COMMUNICATION

Growth hormone regulates intestinal ion transport through a modulation of the constitutive nitric oxide synthase-nitric oxide-cAMP pathway

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secretion, are mediated at an intracellular level by the activity of cNOS.

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Key words: Nitric oxide synthase; Cholera toxin; Intestinal ion secretion; cAMP; Enterocyte

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<http://www.wjgnet.com/1007-9327/12/4710.asp>

Abstract

AIM: Growth hormone (GH) directly interacts with the enterocyte stimulating ion absorption and reducing ion secretion induced by agonists of cAMP. Since nitric oxide (NO) is involved in the regulation of transepithelial ion transport and acts as a second messenger for GH hemodynamic effects, we tested the hypothesis that NO may be involved in the resulting effects of GH on intestinal ion transport.

METHODS: Electrical parameters reflecting trans-epithelial ion transport were measured in Caco-2 cell monolayers mounted in Ussing chambers and exposed to GH and cholera toxin (CT) alone or in combination, in the presence or absence of the NO synthase (NOS) inhibitor, N ω -nitro-L-arginine methyl ester (L-NAME). Similar experiments were conducted to determine cAMP and nitrite/nitrate concentrations. NOS expression was assayed by Western blot analysis.

RESULTS: L-NAME causes total abrogation of absorptive and anti-secretory effects by GH on intestinal ion transport. In addition, L-NAME was able to inhibit the GH-effects on intracellular cAMP concentration under basal conditions and in response to CT. GH induced a Ca²⁺-dependent increase of nitrites/nitrates production, indicating the involvement of the constitutive rather than the inducible NOS isoform, which was directly confirmed by Western blot analysis.

CONCLUSION: These results suggest that the GH effects on intestinal ion transport, either under basal conditions or in the presence of cAMP-stimulated ion

INTRODUCTION

Intestinal ion fluxes are regulated by several agents including neurotransmitters, hormones, or paracrine agents^[1]. We obtained evidence that growth hormone (GH) and nitric oxide (NO) act as modulators in this network^[2-5]. GH increases basal intestinal water and ion absorption in *in vivo* and *in vitro* animal models and is also capable of substantially reducing ion secretion induced by agonists of cAMP, cGMP, or intracellular Ca²⁺, the second messengers of ion secretion^[4,5]. Using the human intestinal cell line Caco-2, we showed that the GH effects on ion transport result from direct interaction with the enterocyte^[2]. Free radical NO acts as a second messenger of several GH effects on human metabolism^[6]. NO production is decreased in patients with untreated GH deficiency, while treatment with recombinant human growth hormone (rhGH) increases NO formation^[7]. In the past decade NO has emerged as a signalling molecule mediating a broad spectrum of intestinal processes, such as gastrointestinal motility, inflammatory changes, malignancy, mucosal blood flow and transepithelial ion transport^[8,9]. NO is a gas with a half life of less than 5 s generated through a series of regulated electron transfer steps by a family of P450-like enzymes, termed nitric oxide synthases (NOS)^[10,11]. Two NOS are continuously present and are termed constitutive nitric oxide synthase (cNOS). These two isoforms are Ca²⁺/calmodulin-dependent, produce small amounts of NO in

short bursts and are involved in homeostatic processes. A third isoform, which is Ca^{2+} /calmodulin-independent, is induced by intestinal injury and inflammation. This latter isoform, termed inducible nitric oxide synthase (iNOS), requires a lag period of at least 2–3 h and, once expressed, produces large amounts of NO for longer time^[13,12]. NO can be directly produced by enterocytes through both the constitutive and the inducible NOS isoforms^[3,13,14]. An important feature of the NO effect is its concentration-dependence. Leading to the concept that NO often acts as a double-edged sword mediator with beneficial as well as detrimental effects. While at lower concentrations it maintains a basal ion intestinal pro-absorptive tone, it increases in several pathologic states such as inflammatory bowel diseases, toxic megacolon, and infectious gastroenteritis, contributing to ion secretion^[8,12,15]. Recently, we showed that under basal conditions the intracellular cAMP concentration ([cAMP]_i) is downregulated in the enterocyte by a cNOS-dependent NO production. Furthermore, in the presence of a cAMP-dependent stimulated secretion, cNOS is activated functioning as a breaking force of ion secretion^[3]. This raised the hypothesis that the enterocyte is capable of self-regulating its own ion transport process through the activation of the cNOS-NO pathway which is able to modulate the [cAMP]_i level^[3]. The aim of this study was to determine whether NO is also involved in mediating the ion absorptive effects triggered by an extracellular stimulus. Specifically, we tested the hypothesis that the cNOS-NO-cAMP pathway is implicated in the pro-absorptive and in the anti-secretory effect induced by GH at the intestinal level. We used the Caco-2 *in vitro* cell model, previously validated for investigating the GH and the NO intestinal effects^[2].

MATERIALS AND METHODS

Cell culture

Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) with high glucose concentration (4.5 g/L) supplemented with 10% FCS, 1% nonessential amino acids, penicillin (50 mU/mL), streptomycin (50 mg/mL) and were incubated in 50 mL/L CO₂-950 mL/L air. Medium was changed daily.

Ion transport studies

Cells were grown on uncoated polycarbonate transwell filters as previously described and used for intestinal transport studies at 15 d post-confluence^[3]. The filter area was 4.9 cm². Each filter was mounted in an Ussing chamber (World Precision Instrument, Sarasota, FL) as a flat sheet between the mucosal and the serosal compartment. Each compartment contained 10 mL of Ringer's solution with the following composition (in mmol/L): NaCl (114), KCl (10), Na₂HPO₄ (1.65), NaH₂PO₄ (0.3), CaCl₂ (1.25), MgCl₂ (1.1), NaHCO₃ (15), glucose (19). In experiments performed to investigate the role of Cl⁻ in the electrical response, SO₄⁻ substituted Cl⁻ at an equimolar concentration. The incubation fluid was circulated by a thermostat-regulated circulating pump and continuously gassed with

95% O₂-5% CO₂. Transepithelial potential difference (PD), short-circuit current (I_{sc}) and tissue ionic conductance (G) were monitored by an automatic voltage-clamp device (DVC 1000, World Precision Instrument, Sarasota, FL) as described elsewhere^[16], before and after mucosal or serosal addition of GH, cholera toxin (CT), and the specific NOS inhibitor N ω -nitro-L-arginine methyl ester (L-NAME). I_{sc} is expressed as microamperes per square centimeter ($\mu\text{A}/\text{cm}^2$), G as millisiemens per square centimeter (mS/cm^2), and PD as millivolts (mV). Caco-2 cell monolayers, pre-incubated for 20 min with GH (4×10^{-9} mol/L) on the serosal side, were exposed to CT (6×10^{-8} mol/L) on the mucosal side, in the presence or the absence of L-NAME (2×10^{-4} mol/L) added to both sides. The maximal effective concentrations of GH, CT and L-NAME were determined by dose-response experiments (data not shown). Cell viability was evaluated at the end of each experiment by measuring the electrical response to the serosal addition of theophylline (5×10^{-3} mol/L).

Intracellular cAMP concentration determination

To test the hypothesis that GH specifically counteracts the CT-induced cAMP increase, we determined the modifications in [cAMP]_i after 1 h of incubation with GH and CT, alone or in combination, and in the presence or the absence of L-NAME. [cAMP]_i in Caco-2 cells which was measured by using a commercial kit (Biotrak cyclic AMP assay system; Amersham International, Amersham, UK), as previously described^[17].

Western blot analysis

Caco-2 cells were stimulated with GH (4×10^{-9} mol/L) for 1, 6 or 24 h. Cells were then scraped into PBS buffer and lysed in a buffer containing 1% Tergitol (Nonidet P-40) with the following composition: KCl, 60 mmol/L; β -mercaptoethanol, 14 mmol/L; EDTA, 2 mmol/L; HEPES pH 7.9, 15 mmol/L; sucrose, 0.3 mol/L; aprotinin, 5 $\mu\text{g}/\text{mL}$; leupeptin, 10 $\mu\text{g}/\text{mL}$; pepstatin, 2 $\mu\text{g}/\text{mL}$; phenylmethylsulfonyl fluoride, 0.1 mmol/L. Whole cellular extracts were centrifuged at 1500 g for 20 min at 4°C. Protein content was determined by the Bradford method (Bio-Rad Laboratories, Munchen, Germany). The supernatant containing the solubilized proteins was then boiled for 5 min in Laemly buffer (tris-HCl pH 6.8, 62.5 mmol/L; SDS 2%; glycerol 10%; 2-mercaptoethanol 5%; bromophenol blue 0.001%). Cell proteins (50 $\mu\text{g}/\text{lane}$) were added to an SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto a nitrocellulose membrane (BioBlot-NC-Costar, Corning Incorporated, Canada). Blots were blocked with T-TBS buffer (tris-HCl pH 8.8, 10 mmol/L; NaCl, 150 mmol/L; Tween 20, 0.05%) containing 3% albumin, and probed for 1 h with affinity purified anti-human NOS1 (1:2000) dilution ratio, NOS2 (1:200) or NOS3 (1:1000) rabbit polyclonal antibodies. Bound antibody was detected with anti-rabbit immunoglobulin horseradish peroxidase-linked whole antibody and developed by chemiluminescence reaction (Amersham Pharmacia Biotech, UK). Gamma-interferon (50000 U/mL) was used as a positive control in experiments performed using anti-iNOS antibodies.

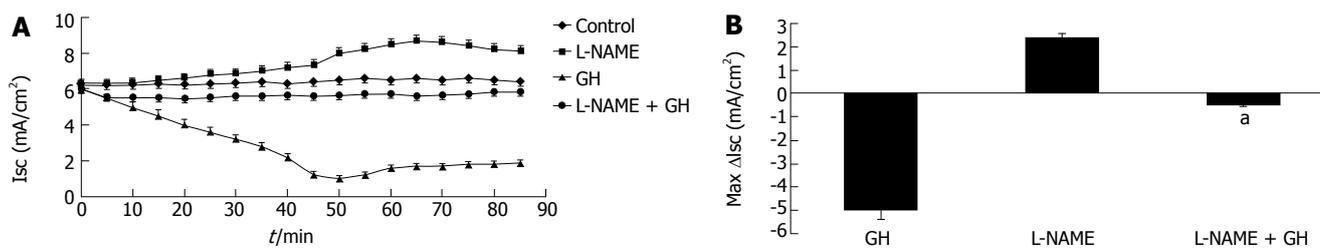


Figure 1 A: Time course of the effect on short-circuit current (Isc) of GH (4×10^{-9} mol/L) and L-NAME (2×10^{-4} mol/L), alone or in combination, to Caco-2 cells mounted in Ussing chambers; B: Isc peak after L-NAME or GH addition, alone or in combination, to Caco-2 cells mounted in Ussing chambers. Data are mean \pm SD of 6 different observations. ^a $P < 0.05$ GH vs L-NAME + GH.

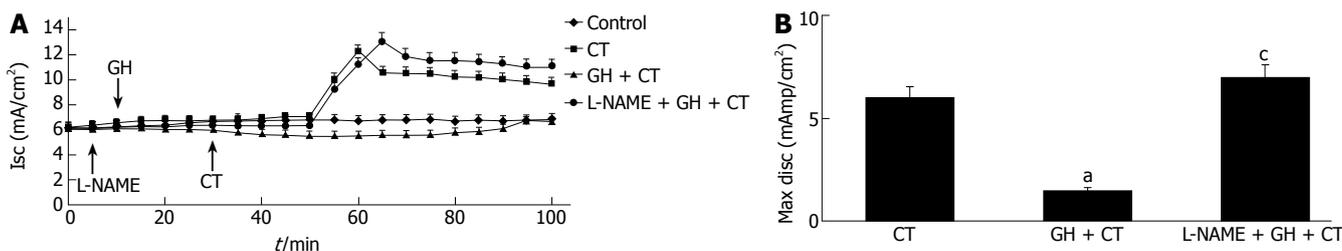


Figure 2 A: Time course of the GH (4×10^{-9} mol/L) effect on CT (6×10^{-8} mol/L)-induced short-circuit current (Isc) increase in the absence or in the presence of L-NAME (2×10^{-4} mol/L) in Caco-2 cells mounted in Ussing chambers. The arrows indicate the time of addition of each agent; B: Maximal Isc increase after CT addition, alone or in the presence of GH alone or in combination with L-NAME. A total abrogation of the antagonistic effect of GH on the CT-induced electrical response was observed in the presence of L-NAME. Data are mean \pm SD of 6 different observations. ^a $P < 0.05$ CT alone vs GH + CT; ^c $P < 0.05$ GH + CT vs L-NAME + GH + CT.

Nitrite/nitrate ($\text{NO}_2^-/\text{NO}_3^-$) production

The combined concentration of nitrite and nitrate, the degradation products of NO in the culture medium, was determined by the Griess reaction after nitrate reduction^[18]. Total nitrite/nitrate production was referred to NO production. Experiments were performed using normal or Ca^{2+} -free Ringer's solution to investigate the involvement of the cNOS isoform (the Ca^{2+} /calmodulin-dependent isoform or NOS1). The modified Ringer's solution had the following composition (mmol/L): NaH_2PO_4 , 1.65; NaH_2PO_4 , 0.3; NaHCO_3 , 15; NaCl , 53; KCl , 10; Na_2SO_4 , 30.5; MgCl_2 , 2.35; glucose, 19; EDTA, 0.5.

Chemicals

All chemicals were of reagent grade and were obtained from Sigma Chemicals Co. (St. Louis, MO, USA). Culture media were from Life Technologies GIBCO BRL (Mascia e Brunelli, Milan, Italy). Transwell filters and supports were from Costar (Costar Italia, Milan, Italy). rhGH was obtained from Serono (Industria Farmaceutica Serono, Rome, Italy). Anti-cNOS and anti-iNOS polyclonal antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-iNOS polyclonal antibodies were purchased from Transduction Laboratories (ABD Company, Lexington, KY, USA).

Statistical analysis

Each experiment was run in duplicate and repeated at least 3 times. Results are expressed as mean \pm SD. Repeated-measures ANOVA were applied using the Bonferroni test for multiple comparisons. The significance was set at 5%. The SPSS software package for Windows (release 11.0.1;

SPSS Inc., Chicago, IL, USA) was used for statistical analysis.

RESULTS

Intestinal transport studies

GH (4×10^{-9} mol/L) and L-NAME (2×10^{-4} mol/L) caused opposite effects on basal Isc. GH induced a decrease and L-NAME induced an increase in Isc. Both effects were totally related to PD modifications, as no significant variations of G were recorded. Pre-treatment with L-NAME (2×10^{-4} mol/L) for 5 m almost abolished the electrical response to GH (Figure 1). The addition of CT (6×10^{-8} mol/L) to Caco-2 cells induced an increase in Isc. Both the GH and CT effects were Cl^- -dependent as demonstrated in the experiment done in Cl^- free Ringer solution. Thus, in the absence of Cl^- the electrical effects were virtually abolished indicating that they were entirely due to transepithelial Cl^- transport modifications (data not shown). Pre-incubation with GH for 20 m substantially reduced the CT effect on Isc. However, pre-incubation with L-NAME resulted in total abrogation of the antagonistic effect of GH on the CT-induced electrical response (Figure 2).

Intracellular cAMP concentration determination

Incubation with GH (4×10^{-9} mol/L) resulted in significant reduction of basal [cAMP]_i. On the contrary, incubation with CT induced an increase in [cAMP]_i. The addition of L-NAME produced a significant increase in basal and in CT-stimulated [cAMP]_i. Pre-incubation with GH for 20 min resulted in a reduction of the CT-induced [cAMP]_i increase. Finally, the addition of L-NAME resulted in total inhibition of the GH-induced decrease in basal [cAMP]_i as

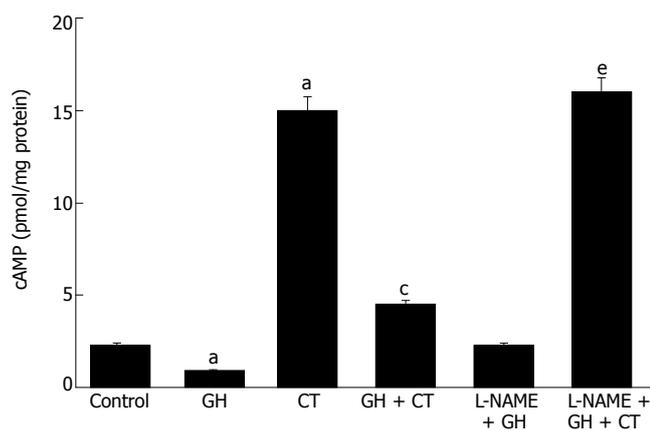


Figure 3 Modification of intracellular cAMP concentration in Caco-2 cells after incubation with GH, CT, alone or in combination, in the presence or in the absence of L-NAME. Data are mean \pm SD of 6 different observations. ^a $P < 0.05$ vs control; ^c $P < 0.05$ CT alone vs GH + CT; ^e $P < 0.05$ GH + CT vs L-NAME + GH + CT.

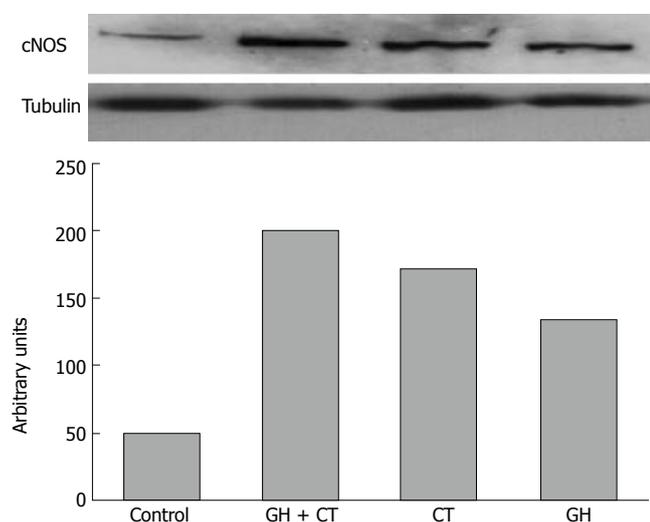


Figure 4 The upper side of the figure shows the cNOS protein expression in Caco-2 cells after 1 h of incubation with GH and CT alone or in combination, as compared to tubulin expression. The cNOS protein expression is revealed by the appearance of 160-kD band that corresponds to human NOS 1 (neuronal NOS). Shown is a representation of 3 separate experiments. In the lower side of the figure an optical densitometry analysis of the bands is also reported.

well as a total abrogation of the GH effect on CT-induced [cAMP]_i increase (Figure 3). These results point to a role of NO either under basal conditions or in response to external stimuli which drive ion fluxes toward an absorption pattern.

Western blot analysis

Caco-2 cells showed low but detectable basal cNOS protein expression (Figure 4). Western blot analysis performed after 1 h of incubation with GH revealed the amplification of a 160 kD band corresponding to human NOS 1. Simultaneous incubation of Caco-2 cells with either GH or CT resulted in further amplification of the NOS 1 band (Figure 4). On the contrary, NOS 2 and NOS 3 protein expressions were undetectable in unstimulated cells and in cells exposed to GH and CT alone or in combination for up to

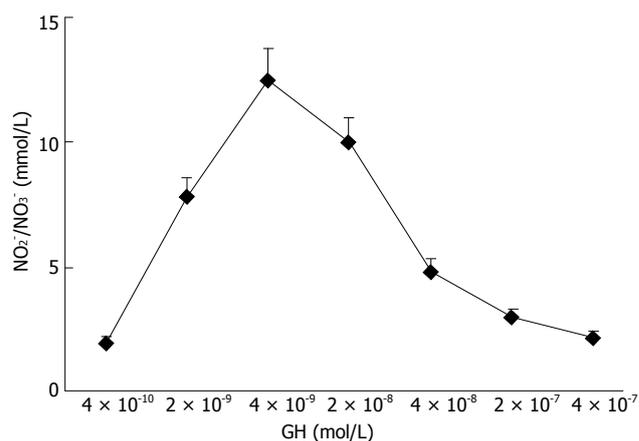


Figure 5 Effects of increasing concentration of GH on NO production in Caco-2 cells. Increasing concentrations of GH were added to Caco-2 cell monolayers and NO production was determined after 1 h of incubation. Data are mean \pm SD of 6 different observations.

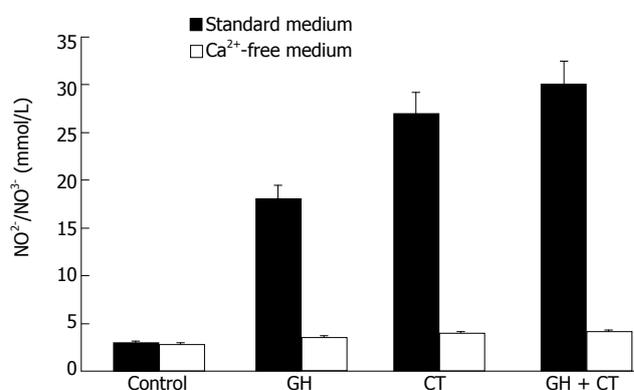


Figure 6 Total NO production in Caco-2 cells under basal conditions and after stimulation with GH and CT, alone or in combination, in standard or in Ca²⁺-free medium. Data are mean \pm SD of 6 different observations. ^a $P < 0.05$ vs control.

24 h of incubation (data not shown).

Nitrite/nitrate (NO₂⁻/NO₃⁻) production

NO production by Caco-2 cells was determined in culture medium after 1 h of incubation with increasing GH doses. As shown in Figure 5 a dose-dependent increase in NO production was detected in response to the hormone. GH doses higher than 4 × 10⁻⁹ mol/L did not induce further increase in NO production, indicating a saturation pattern of the effect. Caco-2 cell stimulation with simultaneous exposure to GH and CT resulted in a further increase in NO production compared to each individual substance. The effect was Ca²⁺-dependent, since in the absence of Ca²⁺, the NO increase in response to GH addition was abolished. This data suggests an involvement of the constitutive rather than the inducible NOS isoform in the GH effect (Figure 6).

DISCUSSION

We have previously shown that GH is able to increase intestinal fluid absorption under basal conditions and to inhibit ion secretion elicited by the 3 main intracellu-

lar second messengers of bacterial enterotoxins: cAMP, cGMP and Ca^{2+} ^[2,4]. The data from this study provides new evidence on the ability of GH to regulate water and ion transport and implicates cNOS-NO activity for this effect. A complete abrogation of GH effects on Isc was seen in the presence of the specific NOS inhibitor L-NAME. An increase of cNOS activity and a subsequent Ca^{2+} -dependent production of NO were observed in enterocytes treated with GH. These effects were associated with a Cl⁻-dependent decrease in Isc, consistent with an anion absorptive effect. We have recently demonstrated that the CT-enterocyte interaction results in an enhanced NO production. Such an effect may be interpreted as a homeostatic mechanism operated by the enterocyte and functioning as a breaking force to limit ion secretion^[3]. cNOS-NO system is activated by a yet unknown sensing mechanism and reacts to balance the stimulated secretion. The data in this work support and extend this hypothesis and suggest that the modulation of the cNOS-NO activity could also be dependent on extracellular stimuli control, namely on the GH signal transduction. In all instances, the target of cNOS-NO is cAMP. Our results are similar to those obtained using NO donors, which are able to inhibit forskolin-stimulated cAMP production by adenylyl cyclase (AC) isoforms AC5 and AC6, in both T84 epithelial cells and mucosal scrapings from mouse colon^[19-21], and are in agreement with those previously obtained in isolated cholangiocytes^[22].

It has been recently suggested that GH inhibitory effect on intestinal ion secretion is related to the transactivation of epidermal growth factor (EGF) receptor and the subsequent activation of extracellular signal-regulated kinase (ERK, also known as p 44/42 mitogen activated protein kinase or MAPK) activity^[23]. Interestingly, a NO stimulation through ERK-dependent upregulation of cNOS gene transcription has been recently demonstrated for proinsulin C-peptide in endothelial cells^[24], and similar effects have been shown with the endothelium-derived hyperpolarizing factor (EDHF)^[25]. Thus, it is possible to also hypothesize that the NO-mediated GH effects at the intestinal level could involve a MAPK activation. It also remains to be clarified whether GH effects are mediated by Ca^{2+} . Overall the cNOS-NO system could be viewed as a regulator of ion transport acting on the enterocyte via three distinct patterns: (1) to keep cAMP production at a low level under basal conditions, in order to maintain an intestinal ion pro-absorptive tone; (2) during stimulation of ion secretion, such as that triggered by CT, to reduce ion secretion; (3) in response to extracellular pro-absorptive stimuli, namely acting as second messenger of the GH-induced ion absorption. In all these 3 instances the target of cNOS-NO is cAMP, the effect is Ca^{2+} -dependent and involves Cl⁻ transcellular flux. Thus the cNOS-NO-cAMP pathway plays a key role on the enterocyte fluid absorptive/secretory processes.

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S- Editor Wang J L- Editor Alpini E- Editor Bi L

RAPID COMMUNICATION

Low utility of plasma Nociceptin/orphanin FQ in the diagnosis of hepatocellular carcinoma

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Abstract

AIM: The utility of serum alpha-fetoprotein (α -FP) in the detection of hepatocellular carcinoma (HCC) is questionable. Very high circulating levels of nociceptin/orphanin FQ (N/OFQ), a ligand for a novel opioid receptor, have recently been reported in HCC. The aim of this study was to assess the role of plasma N/OFQ in the diagnosis of HCC arising in patients with liver cirrhosis.

METHODS: Plasma N/OFQ levels were measured by ELISA in 58 patients (28 HCC and 30 liver cirrhosis) and in 25 healthy controls. The values were correlated with clinical and laboratory features including α -FP. Spearman index, biserial correlation coefficient, non parametric combination (NPC) test and discriminant stepwise analysis were used for statistical evaluation of data.

RESULTS: The upper normal limit of nociceptin was 122 pg/mL. Plasma levels above this cut-off were found in 21.4% of patients with HCC, in 23.3% of those with cirrhosis and in 8% of healthy subjects. α -FP serum levels > 200 ng/mL were found in 46.4% of the patients with HCC and in none of those with cirrhosis. No correlation was found between N/OFQ levels and any of the clinical and laboratory features, including α -FP. By NPC test, HCC and cirrhotic patients were different with regard to α -FP ($P = 0.000$) but not in terms of nociceptin ($P = 0.595$). By point biserial correlation, HCC presence was positively correlated with α -FP ($rpb = 0.52$, $P = 0.000$) but not with N/OFQ ($rpb = 0.16$, $P = 0.157$). In a discriminant analysis, α -FP was significant in the Wilks test ($Y = -0.709 + 0.03 \alpha$ -FP) and properly classified

81% of all patients and 61% of HCC. N/OFQ had lower sensitivity, specificity and predictive values than α -FP.

CONCLUSION: Nociceptin is increased in patients with chronic liver disease, independently of the presence of HCC, although the underlying mechanism has yet to be clarified. We conclude it is not a useful marker for HCC.

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Key words: Hepatocellular carcinoma; Nociceptin/orphanin FQ; Liver cirrhosis; Alpha-fetoprotein

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<http://www.wjgnet.com/1007-9327/12/4716.asp>

INTRODUCTION

Nociceptin/orphanin FQ (N/OFQ) is a 17-aminoacid neuropeptide with selective binding affinity for a novel opioid receptor-like 1 (ORL1/NOP). N/OFQ is synthesized in the neurons of the central and peripheral nervous system and is present in the blood and in the cerebrospinal fluid. The N/OFQ/NOP system has been implicated in a variety of biological functions at both central and peripheral levels: pain modulation, immunity, memory, learning, feeding, locomotion, thermoregulation and activities of the gastrointestinal, cardiovascular, renal and respiratory systems^[1,2]. However, its precise biological role in humans has not been fully defined. The presence of N/OFQ/NOP has also been reported in human neuroblastoma cell-lines^[3]. N/OFQ is detectable in the blood and high circulating levels have been reported in incidences of acute and chronic pain, where they are correlated with the duration of symptoms^[4]. In Wilson disease, probably in relation to altered catabolism caused by liver and/or brain deposits of copper^[5], and in cirrhosis of the liver^[6] slightly increased circulating levels of N/OFQ have recently been found. Moreover, very high

levels have been reported in patients with hepatocellular carcinoma (HCC) suggesting that plasma N/OFQ level might represent a specific marker for HCC^[6].

HCC is the leading cause of death in patients with cirrhosis of the liver^[7]. The most commonly used serological marker for HCC surveillance, alpha-fetoprotein (α -FP), has highly variable sensitivity and specificity^[8] while the diagnostic utility of other markers, such as des- γ -carboxyprothrombin^[9], human hepatocyte growth factor^[10], and serum chromogranin-A^[11] remains unclear.

The aim of this study was to investigate the utility of plasma N/OFQ levels in the diagnosis of HCC in patients with liver cirrhosis.

MATERIALS AND METHODS

Patients and healthy subjects

Fifty-eight consecutively observed patients, 28 with HCC and 30 with liver cirrhosis, and 25 healthy members of the medical staff were included in the study. Informed written consent was obtained from patients and healthy controls. The diagnosis of HCC was based on imaging findings and/or histological confirmation. The diagnosis of liver cirrhosis was clinical and/or histological. The demographic and clinical characteristics of the populations studied are shown in Table 1.

Parameters examined

Clinical features (age, gender, etiology, Child-Pugh score, HCC size, presence of pain) and laboratory parameters as reported in Table 2 (plasma N/OFQ, serum α -FP, blood glucose, blood urea, serum creatinine, K, Na, Ca, bilirubin, aspartate aminotransferase, alanine aminotransferase, gamma glutamyl transpeptidase, alkaline phosphatase, lactic dehydrogenase, creatine phosphokinase, amylases, cholinesterase, albumin, erythrocyte sedimentation rate, C-reactive protein, fibrinogen, international normalized ratio, hemoglobin, red blood cells, white blood cells, platelets) were evaluated through statistical analysis.

N/OFQ assay

Blood drawn from fasting subjects between 7.30 and 9.30 AM was collected in tubes containing K-EDTA. Aprotinin was immediately added to inhibit proteases. Plasma samples, stored at -80°C for less than one month, were loaded onto C-18 containing Sep-columns (Phoenix Europe GmbH, Karlsruhe, Germany), washed with 0.1% trifluoroacetic acid, eluted with 60% acetonitrile in 1% trifluoroacetic acid and freeze-dried. ELISA assay of N/OFQ was performed using a commercially available kit (Phoenix Europe GmbH, Karlsruhe, Germany). Briefly, the lyophilized samples reconstituted with assay buffer were placed in microwells, together with rabbit anti-orphanin FQ serum and biotinylated peptide. After 2 h incubation at room temperature, streptavidin-horseradish peroxidase was added followed by a 1 h incubation. After washing, 100 μ L of substrate solution was added and the reaction was terminated after 1 h with 100 μ L of 2N HCl. Absorbance was read at 450 nm and results were compared to a standard curve ranging from 10 to 100.000 pg/mL.

Table 1 Clinical-Demographic characteristics of the populations studied

	HCC	Cirrhosis	Controls
Number of patients	28	30	25
Mean age \pm SD (yr)	70 \pm 4.8	65.3 \pm 11.1	35.4 \pm 13.9
Male/Female	20/8	21/9	10/15
Etiology <i>n</i> (%)			
HCV	24 (85.8)	15 (50)	
HBV	-	3 (10)	
Alcohol	2 (7.1)	8 (26.7)	
Cryptogenic	2 (7.1)	4 (13.3)	
Child - Pugh score <i>n</i> (%)			
A	22 (78.6)	19 (63.3)	
B	6 (21.4)	7 (23.3)	
C		4 (13.3)	
Patients with pain (%)	4 (14.3)	5 (16.6)	6 (24)
HCC diameter min-max (cm)	1.2-12.5		

HCV: Hepatitis C virus; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma.

Statistical analysis

Correlations among the variables studied were calculated using non-parametric Spearman index and biserial correlation coefficient^[12], as appropriate. Differences between groups were evaluated using the Non Parametric Combination Test, NPC^[13]. $P < 0.05$ was considered as statistically significant. Variables discriminating the groups of patients were identified with discriminant stepwise analysis (Wilks test and Classification results)^[14]. The upper normal limit of N/OFQ was defined as the 95th percentile of the values in healthy controls. Software packages used were Methodologica S.R.L. (2001) for non-parametric analysis NPC test, Confidence Interval Analysis (CIA) Windows version 2.0 (2000) for sensitivity and specificity evaluation, and SPSS, Windows 11.0 (2001) for correlation index and discriminant analysis.

RESULTS

The upper normal limit of N/OFQ was 122 pg/mL. Plasma levels above this cut-off were found in 6 patients with HCC (21.4%), in 7 patients with cirrhosis (23.3%) and in 2 healthy subjects (8%). α -FP serum levels > 200 ng/mL, reported in the literature as highly suggestive for HCC^[20], were found in 13 patients with HCC (46.4%) and in none of the patients with cirrhosis (Figure 1).

In all the groups, N/OFQ levels were not correlated with any of the demographic-clinical features nor with the laboratory parameters mentioned in the materials and methods section. In particular, N/OFQ levels were not correlated with α -FP levels.

The NPC test showed that patients with HCC and patients with cirrhosis were significantly different with regard to α -FP ($P = 0.000$), but not according to N/OFQ levels ($P = 0.595$). Point biserial correlation showed that HCC presence was positively correlated with the levels of α -FP ($rpb = 0.52$ $P = 0.000$) but not with those of N/OFQ ($rpb = 0.16$ $P = 0.157$). Within the discriminant

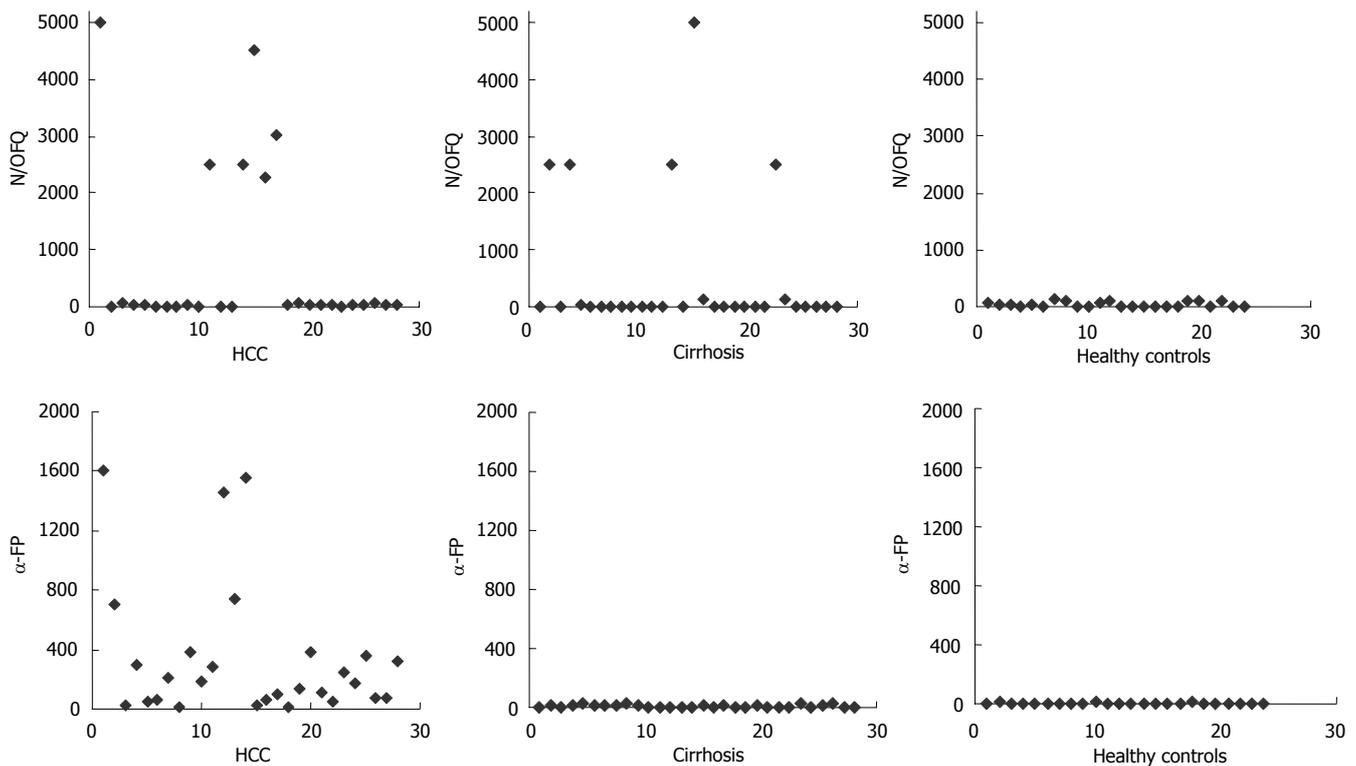


Figure 1 N/O/FQ (pg/mL) and α -FP (ng/mL) values in patients with HCC, with cirrhosis and in healthy controls.

Table 2 Laboratory parameters included in the statistical analysis

Mean values \pm SD	HCC	Cirrhosis	Controls
α -FP (ng/mL)	346 \pm 461 ^b	9.2 \pm 8	3.9 \pm 0.4
N/O/FQ (pg/mL)	728 \pm 1460	515 \pm 1205	138 \pm 493
Aspartate aminotransferase (U/L)	60 \pm 28.5	54 \pm 35	26 \pm 9
Alanine aminotransferase (U/L)	37 \pm 15	53 \pm 50.5	33 \pm 10.5
γ -glutamyl transpeptidase (U/L)	111 \pm 58	78 \pm 60	28 \pm 11
Alkaline phosphatase (U/L)	325 \pm 113.5	285 \pm 185	122 \pm 75
Cholinesterase (U/L)	3695 \pm 1910	4105 \pm 2891	9680 \pm 2899
Bilirubin (mg/dL)	1.6 \pm 1.3	3.7 \pm 7	0.5 \pm 0.3
Albumin (g/dL)	3.2 \pm 0.4	3.4 \pm 0.6	3.7 \pm 0.4
Lactic dehydrogenase (U/L)	418 \pm 53.5	347 \pm 89	263 \pm 78
Creatine phosphokinase (U/L)	136 \pm 61	72 \pm 18	87 \pm 52
Amylases (U/L)	107 \pm 19	90 \pm 33	76 \pm 45
Blood urea (mg/dL)	44 \pm 16	71 \pm 42.5	32 \pm 11
Creatinine (mg/dL)	1.1 \pm 0.3	1.2 \pm 0.5	0.9 \pm 0.2
Na (mmol/L)	135 \pm 3.8	136 \pm 6.8	139 \pm 5.1
K (mmol/L)	4.4 \pm 0.3	4.5 \pm 0.5	4.3 \pm 0.5
Ca (mg/dL)	9.1 \pm 0.5	8.7 \pm 0.4	4.9 \pm 0.3
Red blood cells ($10^6/mm^3$)	3.8 \pm 0.6	1.9 \pm 1.7	4.2 \pm 0.2
With blood cells ($10^3/mm^3$)	4.9 \pm 2	5 \pm 1.8	7 \pm 1.3
Hemoglobin (g%)	11.7 \pm 1.5	10.6 \pm 2.2	13.6 \pm 0.9
Platelets ($10^3/mm^3$)	116 \pm 48	99 \pm 47	249 \pm 58
Blood glucose (mg/dL)	96 \pm 14	111 \pm 38	78 \pm 11
Erythrocyte sedimentation rate (mm)	66.6 \pm 17.7	49.4 \pm 17.1	7.4 \pm 4.1
C-reactive protein (mg/L)	18.4 \pm 29.7	10.3 \pm 9.7	2.7 \pm 1.4
International Normalized Ratio	1.22 \pm 0.2	1.31 \pm 0.3	0.8 \pm 0.1
Fibrinogen (mg/L)	288 \pm 103	243 \pm 68	291 \pm 93

^b $P < 0.001$ vs cirrhosis and controls.

Table 3 Diagnostic accuracy tests

	N/O/FQ		α -FP	
Sensitivity % (CI)	21.4	(0.06 - 0.36)	46.4	(0.29 - 0.64)
Specificity % (CI)	76.7	(0.61 - 0.91)	100	(0.88 - 1.00)
Positive predictive value % (CI)	46.2	(0.19 - 0.73)	100	(0.77 - 1.00)
Negative predictive value % (CI)	51.1	(0.36 - 0.65)	66.7	(0.52 - 0.78)
Positive likelihood ratio (CI)	0.92	(0.35 - 2.40)	∞	(4.01 - ∞)
Negative likelihood ratio (CI)	1.02	(0.77 - 1.35)	0.53	(0.33 - ∞)

analysis, the Wilks test showed that only α -FP was significant in the model ($Y = -0.709 + 0.03 \alpha$ -FP). The classification of results demonstrated that α -FP properly classified 81% of all patients and 61% of patients with HCC. Sensitivity, specificity and predictive values of N/O/FQ and α -FP in the diagnosis of HCC are reported in Table 3.

DISCUSSION

HCC is the third leading cancer-related cause of death worldwide^[15]. In western countries, HCC arises in cirrhotic livers with an annual incidence of 3%-5%. Early detection of HCC allows optimal application of curative treatments. The increased survival, after radical treatment in the last decade, recommends surveillance for detection of early HCC in cirrhosis^[16]. Ultrasonography plays a key role in the detection of HCC but its sensitivity for small

nodules is low^[17] and other imaging techniques, such as CT and MRI, are too expensive for screening programs. An elevation in serum α -FP is associated with HCC but it is also elevated in non-hepatic malignancies^[18] and in hepatitis^[19]. Although the diagnostic accuracy of serum α -FP is highly variable in the reported series depending on the cut-off level^[20], it remains the most commonly used serological marker for HCC surveillance. The diagnostic value of other markers proposed so far, such as des- γ -carboxyprothrombin^[9], human hepatocyte growth factor^[10], serum chromogranin-A^[11], is not well defined. It has recently been published that plasma levels of N/OFQ are very high in all patients with HCC, including those with normal α -FP^[6]. With the aim to define if plasma N/OFQ values represent an indicator of HCC even in absence of increased α -FP, we studied circulating N/OFQ and α -FP levels in patients with liver cirrhosis, with and without HCC, comparing the diagnostic value of the two markers.

Mean N/OFQ values were higher both in patients with liver cirrhosis alone and in those with HCC than in controls, but did not differ significantly between the two patient groups, with a similar percentage of values above the normal range. On the other hand, mean α -FP levels in HCC patients were higher than in cirrhotics, with a higher percentage of values > 200 ng/mL. The NPC test showed significant differences between patients with and without HCC with regard to α -FP but not for N/OFQ. Such data are in accordance with the results of Szalay *et al.*^[6] with respect to the increase in plasma N/OFQ values seen in cirrhotic patients, but do not confirm the unique finding of extremely elevated values in the entire population with HCC. Since the demographic and clinical features, including size of the tumor and presence of pain, are similar among the patients enrolled in the two studies, we are not able to suggest any explanation other than possible genetic differences for the discordant results.

Receptors for N/OFQ are normally present in the liver as shown by detection of mRNA for the ORL1/NOP receptor^[21]. Therefore, an increase in plasma N/OFQ values in some patients with chronic liver disease, with and without HCC, might be due to the chronic liver disorder with a reduced ability to bind N/OFQ.

Transcription of the N/OFQ gene is enhanced by estrogen^[22], therefore another explanation for the increase of N/OFQ plasma levels in cirrhotic livers, independently of the presence of HCC, might be the increase of estrogen hormones, common in patients with advanced liver disease.

A higher N/OFQ content has been shown in HCC tissue as compared to tumor-free liver tissue in one patient with PBC^[23] and in rats with experimentally induced tumors^[24], suggesting that the HCC cells might produce N/OFQ or give signals for neuronal production. However, we were not able to find any correlation between plasma N/OFQ levels and tumor size, in agreement with the finding of Szalay *et al.*^[6].

Peripheral blood neutrophils express and secrete N/OFQ following degranulation^[25]; N/OFQ stimulates chemotaxis^[26] and is present at sites of inflammation, such as synovial exudates^[25]. However, in this study, we were not able to correlate the amounts of circulating N/OFQ with

any of the inflammatory indices (erythrocyte sedimentation rate, C-reactive protein, peripheral leukocytes, fibrinogen, platelets).

It has been demonstrated that N/OFQ plays a role in the perception of pain, inhibiting the release of various neurotransmitters, including pain related peptides^[27,28]. However, no correlation between plasma values of N/OFQ and the presence of pain in any of the groups studied was found.

When we compared the diagnostic utility of N/OFQ and α -FP in HCC, α -FP had better sensitivity (46% *vs* 21%), better specificity (100% *vs* 77%), higher positive predictive value (100% *vs* 46%) and negative predictive value (67% *vs* 51%).

In conclusion, from our experience, N/OFQ cannot be recommended as a marker for early detection of HCC in patients with liver cirrhosis. α -FP is very specific when a high cut-off (200 ng/mL) is adopted but its sensitivity is low. N/OFQ is increased in patients with chronic liver disease, independent of the presence of HCC, although the underlying mechanism needs to be clarified. In the diagnosis of HCC, further research is needed in order to find serum markers more sensitive than α -FP.

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CCR5 Δ 32 mutation does not influence the susceptibility to HCV infection, severity of liver disease and response to therapy in patients with chronic hepatitis C

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($P = NS$). There was also no association observed with response to therapy and CCR5 Δ 32 mutation.

CONCLUSION: CCR5 Δ 32 mutation does not have a role in disease susceptibility, severity or response to therapy in patients with chronic hepatitis C infection.

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Key words: β -chemokine receptor 32 bp deletion; CC chemokine ligand; Human leukocyte antigen; Histological activity index

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Abstract

AIM: To study whether CCR5 Δ 32 mutation was associated with viral infection and severity of liver disease.

METHODS: Two hundred and fifty two histologically proven, chronic HCV patients (mean age: 41 \pm 14 years; M/F: 164/88) were genotyped. PCR based genotyping of 32 bp deletion at the CCR5 locus was done. Four-hundred and eight matched healthy controls were studied to assess susceptibility to HCV infection. To assess correlation of immune gene polymorphism with severity of HCV related liver disease, patients with chronic HCV infection were divided into those with a fibrosis score of ≤ 2 (mild) or > 2 (severe) and histological activity index (HAI) of ≤ 5 or > 5 . For correlation between CCR5 Δ 32 mutations and response to therapy, 129 patients who completed therapy were evaluated.

RESULTS: The majority (89.4%) of the patients were infected with genotype 3. The frequency of homozygous CCR5 Δ 32 mutants was comparable to HCV patients as compared to the healthy controls (0.7% vs 0%, $P = 0.1$). Further more, the frequency of CCR5 Δ 32 mutation was comparable in patients with mild or severe liver disease.

INTRODUCTION

HCV is the only known positive stranded RNA virus that causes persistent long-life infection in humans. Hepatitis C virus is an important cause for chronic liver disease and hepatocellular carcinoma in a proportion of patients with persistent infection. The mechanisms of chronicity and progression of liver disease are probably multifactorial and involve a rather complex interplay between the virus and the host. There has been a surge of interest in studying various immunomodulatory genes to answer these questions.

Hepatitis C virus being a hepatotropic virus requires the recruitment of virus specific T cells to the liver for viral clearance. Chemokines and interaction with their receptors may regulate the selective recruitment of primed Th-1 cells to the site of inflammation. It has been proposed that the immune response in chronic hepatitis C is compartmentalized, with a predominantly Th-2 or Th-0 response in the periphery and a Th-1 response in the liver^[1-5]. In contrast, patients who clear the virus have a predominant peripheral Th-1 response. Moreover, differences in chemokine receptor expression between Th-1 and Th-2 cells might explain their selective recruitment to tissue. In particular, lymphocytes infiltrating HCV infected liver express high levels of the chemokine receptor (CCR5) and CXCR4

chemokine receptor (CXCR3). The CC chemokine macrophage inflammatory protein (MIP)-1 α (CCL3), MIP-1 β (CCL4) and RANTES (CCL5) are important in immune surveillance. These chemokines bind to their corresponding receptors CCR1 and CCR5. Both these receptors are preferentially expressed on lymphocytes with a Th-1 cytokine secretion pattern. Chronic hepatitis C virus infection has been shown to lead to reduced surface expression of CCR1 and CCR5 on peripheral blood T lymphocytes^[6] and more so, on CD8 lymphocytes. While the mechanism of altered chemokine receptor expression and chemokine responsiveness is still not clear, reduced expression would probably lead to a decrease in T lymphocyte migration in response to MIP-1 α , MIP-1 β and RANTES in chemotaxis assays.

The Δ 32 mutation of the CCR5 gene was reported to be associated with inflammatory bowel disease by some but not all authors^[7]. Furthermore, a negative correlation with susceptibility to rheumatoid arthritis has been described^[8,9]. In contrast to these weak or contradicting correlations, the Δ 32 mutation was found to be of paramount importance for protection against HIV infection. The β -chemokine receptor CCR5 appears to be the major co-receptor for entry of macrophage-tropic and non-syncytium-inducing (NSI) variants of HIV^[10]. A 32 bp deletion mutation (Δ 32) of the CCR5 gene encodes a non-functional protein. Homozygosity for the Δ 32 deletion makes the CD4 T cells resistant to infection from NSI virus strains or delay HIV-1 disease progression in CCR5 Δ 32 heterozygotes.

There is a controversy related to the role of CCR5 Δ 32 mutation in susceptibility to HCV infection and disease progression. Woitas *et al* have suggested that a genetically determined loss of CCR5 gene expression is linked to chronic HCV infection and high viral load^[11]. On the other hand, a recent study by Promrat *et al* has shown that reduced expression of CCR5 and RANTES may lead to reduced hepatic inflammation and 59029 -G/A to improved response to interferon therapy in chronic hepatitis C^[12]. Hellier *et al* also suggested a possible role of CCR5 Δ 32 polymorphism in the outcome of HCV infection^[13].

The present case-control association study was undertaken to identify whether CCR5 Δ 32 mutation is associated with HCV disease progression and severity. We also compared the allele frequencies defined by CCR5 Δ 32 mutation in HCV patients with a control group of healthy individuals in order to identify a possible association with susceptibility to disease.

MATERIALS AND METHODS

Patients

Two hundred and fifty two patients with histologically proven chronic HCV infection were studied. The *inclusion criteria* were evidence of chronic hepatitis on liver biopsy and HCV RNA positivity on two occasions at baseline. The HCV RNA was detected as described elsewhere^[14]. HCV genotyping was performed with the reverse hybridization line probe assay (LIPA; Innogenetics, Ghent, Belgium). Patients were *excluded* if they had hepatitis B virus (HBV) or HIV infection, history of heavy alcohol consumption

(> 80 g/d for > 5 years), positivity for antinuclear or anti smooth muscle antibody (in 1:80 dilution), autoimmune liver disease, thyroid disease, diabetes mellitus or malaria. The Institutional Ethical Committee approved the study protocol. An informed consent was obtained for enrolling the patients. The clinical and biochemical assessment of the patients was done according to the study protocol. The histological examination of liver biopsies was done according to the modified Knodell scoring system^[15].

Patients received either 3 million IU of IFN α 2b daily for 24 to 48 wk. A group of patients received pegylated IFN-alpha 2b (1 to 1.5 μ g per wk) for either 24 or 48 wk depending on viral genotype. All patients received ribavirin given orally (patients with < 65 kg received 800 mg/d and 65-85 kg received 1000 mg/d). Outcome of treatment was classified as follows: End Therapy Response (ETR): patients with undetectable HCV RNA at the end of treatment; Sustained Virological Response (SVR): patients with undetectable HCV RNA 6 mo after the end-of-treatment; Non-Response (NR): failure to achieve viral clearance at the end-of-treatment or 6 mo there after.

Controls

Unrelated healthy adult subjects, with no previous history of liver disease and negative for HBV and HCV infection were included as controls. A total of 408 healthy subjects were included as controls. Both patients and control groups included Hindus, Muslims, and Christians. Patients and controls were prospectively matched for ethnic group.

DNA extraction and PCR- based genotyping of allelic variants

Genomic DNA was extracted from whole blood using a commercially available kit. The CCR5 Δ 32 deletion mutation was detected by PCR based techniques as mentioned elsewhere^[16]. Primers amplified 2 fragments of 200 bp and 172 bp corresponding to wt and deleted CCR5 alleles. Primer sequences were CCR5L-5' TTA AAA GCC AGG ACG GTC AC 3' CCR5R-5' GAC CAG CCC CAA GAT GAC TA 3'. Cycling conditions were 96°C for 5 min, followed by 30 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s. A final extension step of 72°C for 10 min was applied. Amplified fragments for the CCR5 locus were resolved in 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

DNA sequencing

The CCR5 Δ 32 mutation was confirmed by sequencing. For DNA sequencing, ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (ABI) was used.

Correlation of immune gene polymorphism and persistence of HCV infection

To study whether persistence of HCV infection is linked to immune gene polymorphism, patients with chronic HCV infection were compared with healthy controls.

Correlation of immune gene polymorphism and severity of HCV related liver disease

To study whether immune gene polymorphisms influence the course of HCV related liver disease, patients were

Table 1 Demographic profile of patients with chronic HCV infection and healthy controls

	Patients (n = 252)	Patients (Only genotype 3) (n = 187)	Healthy controls (n = 408)
Age (yr)			
Mean ± SD	40 ± 14	40 ± 14	33 ± 7
Male: Female	164:88	118:69	297:111
ALT (IU/L)			
Mean	112.3 ± 68.4	116 ± 70.1	29 ± 2
Range	(32-330)	(32-330)	
S. Albumin (g/L)	36 ± 4	34 ± 5	40 ± 3
HCV genotype (n = 209)			
I	17 (8.1%)	-	-
II	1 (0.5%)	-	-
III	187 (89.4%)	-	-
IV	4 (1.5%)	-	-
Liver Biopsy			
Fibrosis score	2 ± 1	2 ± 1.2	-
HAI	5.5 ± 2.4	5.6 ± 2.5	-
Mode of Acquisition of HCV			
Blood transfusion	86	-	-
Needle stick injury	54	-	-
Intravenous drug use	10	-	-
Medical procedure	45	-	-
Unclear	57	-	-

categorized on the basis of the stage of ALT levels (< 60 or ≥ 60 IU/L), hepatic fibrosis and histological activity index (HAI). Patients were divided into those with a fibrosis score of ≤ 2 (mild) or > 2 (severe) and with a necroinflammatory score of ≤ 5 (mild) or > 5 (severe).

Statistical analysis

The frequencies of CCR5Δ32 mutant allele were compared between patients with chronic HCV infection and controls by Chi-square test. Categorical variables were analyzed with χ^2 or Fisher's exact test. Two-sample *t*-tests were used to compare means for continuous variables and for non-normally distributed continuous variables, non-parametric test, Wilcoxon Mann-Whitney-U test was used for comparison of median values. Univariate analysis was used to assess associations between the various allelic variants and severity of liver disease.

Hardy-Weinberg equilibrium was tested by comparing expected and observed genotype frequencies by χ^2 -test. The distribution of genotypes between the patients and healthy controls were compared by contingency table analysis.

For all tests, a 2-tailed *P* < 0.05 was considered significant. The analysis was performed by using statistical software SPSS 12.0 software (SPSS, Chicago, IL).

RESULTS

Demographic profile

Two hundred and fifty two patients with chronic hepatitis C were studied. The baseline characteristics of the cases and controls in the study group are summarized in Table 1. The most common source of HCV infection was blood transfusion.

Table 2 Allele frequency of CCR5 variants in chronic HCV patients and healthy controls

CCR5	HCV Patients (n = 252)	Healthy Controls (n = 408)	<i>P</i> value
Wt/wt	247 (98%)	405 (99.3%)	0.07
Δ32mt carrier	5 (1.9%)	3 (0.7%)	0.3
Wt/Δ32	3 (1.2%)	3 (0.7%)	0.2
^a Δ32mt/Δ32mt	2 (0.7%)	-	0.1

^aThe patients were genotype 1.

Viral genotype

HCV genotypes could be determined in 209 of the 252 (82.9%) patients. The predominant viral genotype was genotype 3a/3b. One hundred eighty seven (89.4%) patients had genotype 3, 17 (8.1%) had genotype 1, one patient had genotype 2 and 4 patients had genotype 4 (Table 1). No significant difference was observed in immune gene polymorphism and patients with different viral genotypes.

Distribution of CCR5 allelic variants

The frequency of CCR5Δ32 mutations in HCV patients and controls is shown in Table 2. Two hundred and forty seven (96.6%) HCV patients and 405 (99.3%) healthy controls had CCR5 wt/wt homozygosity. 3 (1.2%) of 252 patients had heterozygous and 2 (0.7%) had homozygous Δ32 mutations; overall 5 (1.9%) of the HCV patients had Δ32 mutant alleles. On the other hand, none of the healthy controls had a homozygous Δ32mt/Δ32mt pattern. Three healthy controls exhibited heterozygosity (Table 2). The difference between the patients and the controls was comparable but not significant. CCR5Δ32/Δ32 was present in HCV genotype 1 patients. Patients carrying CCR5Δ32 (CCR5Δ32 homozygote and heterozygote) mutation had no significant difference in the serum ALT level, degree of hepatic inflammation, fibrosis score and viral genotype compared with those patients who had wild type CCR5. All the allelic variants were in Hardy-Weinberg equilibrium.

Relation to serum alanine aminotransferase

We also used the mean serum ALT concentrations as an indicator of the activity of liver disease. However, no significant difference was observed for any of the genetic markers in patients with normal or without raised ALT (data not shown).

Relation to liver histology: Fibrosis and Histological inflammation

When patients were categorized based on the severity of hepatic fibrosis, 162 patients had mild and 90 patients had severe hepatic fibrosis. The frequency of CCR5Δ32 mutations was comparable in patients with mild or severe liver disease (*P* = NS) and it did not correlate with the severity of the liver disease (Table 3). Similarly CCR5Δ32 mutations did not correlate with histological severity

Response to therapy

Table 4 depicts the response pattern of the patients

Table 3 Correlation of Immune Gene Polymorphism and histological severity of HCV Related Liver Disease in Univariate analysis

Liver histology	CCR5-Genotype			P
	wt/wt	wt/ Δ 32	Δ 32/ Δ 32	
Fibrosis				
≤ 2 (n = 162)	154 (98%)	1 (0.6%)	2 (1.2%)	NS
> 2 (n = 90)	88 (97.8%)	2 (1.2%)	0	
HAI				
≤ 5 (n = 162)	170 (98.3%)	1 (0.6%)	2 (1.2%)	NS
> 5 (n = 90)	77 (97.5%)	2 (2.5%)	0	

enrolled in the study. When patients were categorized on the basis of the response pattern, no difference was observed in the distribution of alleles with respect to response rate. Of 252 patients, 129 patients received antiviral therapy. Eighty six patients had attained sustained virological response, 34 patients were non-responders while 7 patients relapsed after discontinuation of therapy and one patient developed decompensation and discontinued the therapy. Two patients who were CCR5 Δ 32/ Δ 32, did not receive therapy. There was also no difference in the response pattern among the heterozygotes.

DISCUSSION

Host genetic factors encoding for gene products, which are likely to be involved in the immune response following HCV infection, are likely to influence the disease susceptibility and progression. It has been demonstrated by studies which correlated polymorphisms or mutations of genes encoding for HLA subtypes^[17-19], tumor necrosis factor^[20-22], interleukin 10^[23] or chemokine receptor 5 (CCR5) with disease susceptibility or treatment outcome in HCV.

In our population 1.9% (5/252) HCV patients were carriers of the CCR5 Δ 32 mutation as compared to 0.7% (3/408) of the general population. This refers to both homozygous and heterozygous mutations combined. Since the frequency of these mutations is rather low it is difficult to make definite statistical analysis between various groups. The reason for the variance in our results compared to the earlier studies is not easy to explain. This could probably be because the frequency of these mutations is rather low, thus it is difficult to make definite statistical analysis between various groups. An earlier study from India has reported a single heterozygote for CCR5 Δ 32 mutants^[24]. However, the group had only genotyped 150 normal healthy individuals for the presence of Δ 32 allele. We had adequate number of controls (n = 408). Woitas *et al* had observed a higher frequency of CCR5 Δ 32 homozygotes in chronic HCV patients^[11]. In the present study, we observed a 0.7% occurrence of CCR5 Δ 32 mutants in chronic HCV patients but none in healthy controls, however the difference was not significant.

Apart from the sample size, a plausible explanation for these divergent results might be the differences in comorbidity of patients in the studies by Woitas and Nguyen^[11,25], who had a substantial proportion of patients with haemophilia and/or concomitant hepatitis B. In the present study

Table 4 Response patterns in HCV patients genotyped for CCR5

Response	CCR5-Genotype			P
	wt/wt	wt/ Δ 32	Δ 32/ Δ 32	
IFN+ Riba/ Responders	86	1	-	NS
Peg IFN+ Rib Non-responders	34	1	-	NS
Relapsers	7	-	-	NS

patients with haemophilia, hepatitis B or HIV were carefully excluded. Moreover, we did not detect elevated aminotransferase levels in carriers of the Δ 32 mutation, which might also be due to the small number of homozygous carriers of the Δ 32 mutants in the present study^[26].

CCR5 Δ 32 mutants did not increase the likelihood of a more severe liver disease in our patients due to HCV infection (Table 3). Table 5 gives an overview of the published data in chronic HCV patients genotyped for CCR5 Δ 32 mutation. As shown in Table 5, the correlation of CCR5 Δ 32 mutations with histological severity is controversial. Significant associations were found between CCR5 Δ 32 and reduced portal inflammation and milder fibrosis^[13]. Liver inflammatory activity was found to be significantly reduced in Jewish Israeli patients infected with the hepatitis C virus carrying the CCR5 Δ 32 allele^[32]. Heterozygosity for CCR5 Δ 32 has been shown to be significantly associated with lower hepatic inflammatory scores^[26]. However, other studies failed to find an association of CCR5 Δ 32 mutations with the histological severity.

Table 5 also shows distribution of viral genotypes in the published data for CCR5 Δ 32 mutation. It is known that the distribution of genotypes could be dependent on geographical distribution and possibly susceptibility. In all the previous studies, the associations have been studied primarily in the population of HCV patients infected with genotype 1. It might be possible that this could influence the susceptibility and severity of liver disease and could have led to variations in the results. The present study is the first study which has looked into the role of CCR5 Δ 32 mutation in genotype 3.

In the study population, nearly 161 of 182 (88.4%) patients in whom genotyping could be done, had genotype 3 infection. Most studies from the Indian subcontinent have reported similar frequency of genotype 3^[34]. HCV 3a is a hepatitis virus strain that responds better to interferon IFN- α therapy than other HCV strains. IFN- α induces the production of CCL3, a CCR5 ligand, in the liver. It is thus possible that CCR5 is involved in a cascade of events or recruitment of immune cells, which negatively regulate the production of IFN- α in the liver. Loss of CCR5 expression due to CCR5 Δ 32 mutation could affect the course of hepatitis C, probably by interfering with cellular immune response. It has been suggested that CCR5 Δ 32/ Δ 32 mutant has no expression of CCR5 on the cell surface and henceforth is an ineffective HCV-specific immune response resulting in an immune imbalance to Th-1 \rightarrow Th-2 response^[27].

It is therefore important to study the role of CCR5 Δ 32 mutation with response to therapy. Ahlenstiel *et al* have shown that response rates to interferon-alpha monotherapy are reduced in hepatitis C virus infected patients

Table 5 Analysis of published data in chronic HCV patients genotyped for CCR5Δ32 mutation

Authors	Susceptibility	Histological Severity	Response to therapy	Genotype	Patients studied (n)
Ahlensteil G <i>et al</i> ^[27,28]	Yes	Not mentioned	Lower ETR ¹	Genotype 1 Genotype 2 Genotype 3 Genotype 4 Undetermined	59 3 9 3 4
Glas J <i>et al</i> ^[29]	No	Not mentioned	No	Genotype 1/4 Genotype 2/3	37 25
Goulding C <i>et al</i> ^[26]	² Spontaneous viral clearance	² Lower hepatic inflammation	Not mentioned	Genotype 1b	283
Hellier <i>et al</i> ^[13]	No	Decreased portal inflammation	No	Stratification not mentioned	
Konishi <i>et al</i> ^[30]	No	No	No	Serotype 1 Serotype 2 Serotype 1 + 2	53 48 4
Mascheretti <i>et al</i> ^[31]	No	No	No	Genotype 1 Genotype 2 Genotype 3 Genotype 4	358 14 79 14
Promrat <i>et al</i> ^[12]	No	No	No	Genotype 1 Genotype 2 Genotype 3 Genotype 4 Genotype 5 Undetermined	243 34 16 3 1 42
Wald <i>et al</i> ^[32]	No	Significantly reduced	Not mentioned	Genotype 1 Genotype 2 Genotype 3 Undetermined	5 2 9 31
Wasmuth <i>et al</i> ^[33]	No	No	No	Genotype 1 Non-Genotype 1	213 117
Woitas <i>et al</i> ^[11]	Yes	Not mentioned	Not mentioned	Genotype 1 Genotype 2 Genotype 3 Genotype 4 Multiple genotype Undetermined	95 16 10 6 5 2
Present study Goyal <i>et al</i>	No	No	No	Genotype 1 Genotype 2 Genotype 3 Genotype 4 Undetermined	16 1 161 4 70

¹ All analysis mentioned w.r.t the CCR5Δ32 carriers. ² Heterozygotes for CCR5Δ32 mutation.

carrying the CCR5Δ32 mutation^[28]. It is difficult to comment on the role of CCR5 mutations in HCV infection with genotype 3 in our population, since this is the most common genotype. Also, we had 0.7% allelic frequency of CCR5Δ32 and since 98% of HCV patients had CCR5 wt/wt, speculating a higher SVR in 3a genotype is not possible. Our patients had received combination therapy of interferon/pegylated interferon and ribavirin. However it has been suggested that the interferon and ribavirin combination treatment may overcome this negative effect of CCR5Δ32^[28]. Thus, in population a infected with genotype 3 HCV, CCR5Δ32 mutations do not influence the response to combination therapy with interferon and ribavirin.

In conclusion, our results indicate that CCR5Δ32 mutation does not influence the susceptibility and severity of liver disease in chronic hepatitis C patients. Moreover, CCR5Δ32 mutation does not influence the response to therapy.

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Coimmunization with IL-15 plasmid enhances the longevity of CD8 T cells induced by DNA encoding hepatitis B virus core antigen

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Abstract

AIM: To test the feasibility of delivering a plasmid encoding IL-15 as a DNA vaccine adjuvant for improving the immune responses induced by hepatitis B virus core gene DNA vaccine.

METHODS: We used RT-PCR based strategies to develop IL-15 expression constructs. We first confirmed that the gene could be expressed in *Escherichia coli* due to the poor expression of IL-15. Then the bioactivity of IL-15 plasmid expression product was identified by CTLL-2 proliferation assay. One hundred micrograms of DNA from each of the IL-15 eukaryotic expressed plasmid and the recombinant plasmid harboring DNA encoding the 144 amino acids of the N-terminus of HBV core gene (abbreviated pHBc144) was used to co-immunize C57 BL/6 mice. The titer of anti-HBcIgG was detected by ELISA and the antigen-specific CD8⁺ T cells (CD8⁺IFN- γ ⁺ T cells) were detected by intracellular cytokine staining at different time points.

RESULTS: After co-immunization by pIL-15 and pHBc144 DNA vaccine the antigen-specific CD8⁺ cells of mice increased gradually, the first peak of immune response appeared 14 d later, then the number of antigen-specific CD8⁺ T cells decreased gradually and maintained at

a steady level in 3 mo. After boosting, the number of antigen-specific CD8⁺ T cells reached the second peak 10 d later with a double of the 1st peak, then the number of antigen-specific CD8⁺ T cells decreased slowly. IL-15 as a gene adjuvant had no significant effect on humoral immune responses induced by hepatitis B virus core gene DNA vaccine, but increased the memory antigen-specific CD8⁺ T cells induced by hepatitis B virus core gene DNA vaccine.

CONCLUSION: DNA vaccine constructed by HBc Ag 1-144 amino acid induces effective cell immunity, and cytokine plasmid-delivered IL-15 enhances the longevity of CD8⁺ T cells.

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Key words: Vaccine; DNA vaccine; Hepatitis B virus core antigen

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INTRODUCTION

The final goal of a vaccine is to make body acquire long-term and effective protection. However the long-term and effective protection depends on the quality and quantity of memory T cells. IL-15 mRNA is constitutively expressed by various cells and tissues such as placenta, skeletal muscle, kidney, epithelial cells, synovial cells, and macrophages^[1]. IL-15 is essential for CD8⁺ memory T-cell generation and maintenance, and is involved in the maintenance of antigen-specific memory CD8⁺ T cells. The number of memory-type (CD44^{high}Ly6C) CD8⁺ T cells increases significantly in the periphery lymphoid tissue of IL-15 Tg mice, producing IL-15, while the number of antigen-specific memory CD8⁺ T cells declines in IL-15⁻ and IL-15R deficient mice^[2].

IL-15 has attracted great attention because of its important effect on memory CD8⁺ T cells^[3-5]. This report describes the IL-15 mRNA extraction from

human peripheral blood mononuclear cells (PBMCs) and the molecular cloning of IL-15. We demonstrated that recombinant IL-15 was expressed in *Escherichia coli* and showed bioactivity in COS cells. Our results suggest that IL-15 plasmid has effect on the memory phase of cellular immune response induced by HBc DNA vaccine, indicating that plasmid IL-15 functions as a candidate adjuvant and can be used in for vaccine or immunotherapeutic studies.

MATERIALS AND METHODS

Mice

Female C57BL/6 mice at the age of 6-8 wk were purchased from Shanghai Sippr-Bk Company and allowed to acclimate for at least 2 wk prior to use. The mice were housed in laminar airflow cages with free access to food and water *ad libitum*. Analyses of specimens from those animals revealed no evidence of exposure to viral, bacterial or parasitic pathogens during these studies.

Plasmids and peptides

The HBcAg-expression plasmid pHBc144 was constructed by inserting a gene encoding hepatitis B core antigen N¹ end 144 amino acid into a vector (pcDNA3, Invitrogen), driven by the cytomegalovirus (CMV) immediate early promoter. The secreted HBcAg could be detected in the supernatant of cell culture transiently transfected with pHBc144^[6]. Anti-HBc could be detected in the serum of Balb/c mice, which were injected with pHBc144 DNA plasmid.

The HBV core Ag peptides were synthesized and generously provided by Dr. Rafi Ahmed (Emory University, GA)

Cell culture

PBMCs were isolated from peripheral blood of healthy volunteers by density gradient centrifugation over Ficoll. Fresh whole blood was drawn, heparinized and gently mixed with one volume of RPMI1640. The diluted blood was layered over Ficoll and centrifuged at 800 g, for 20 min at 4°C. PBMC were collected from the interface between the plasma and the density gradient solution. After being washed in PBS, PBMCs were resuspended in RPMI 1640 supplemented with 5 mg/mL LPS, 200 mmol/L L-glutamine, 10% FCS, 20 mmol/L Hepes, 100 U/mL penicillin, and 100 µg/mL streptomycin, at a final cell concentration of 5×10^6 /mL. Then the PBMCs were cultured in 24-well plates for 6 h.

RT-PCR and cDNA cloning of Interlukin-15

RNA extraction as well as RT-PCR analysis were performed as previously described^[7]. Briefly, total RNA was purified using Trizol (Invitrogen Life Technologies) following the manufacturer's instructions. A 4 µg aliquot of total cellular RNA was reverse transcribed using random hexanucleotide primers and the Superscript II preamplification system (Invitrogen Life Technologies). One-tenth of the cDNA obtained was amplified in a 20-µl reaction, using 1 U of P_{wo} DNA Polymerase Expand¹™ High Fidel-

ity PCR System (Boehringer Mannheim) and the reagents provided with the preamplification system. Thirty-five cycles of PCR amplification were performed at 94°C for 2 min, at 60°C for 1 min and at 72°C for 1 min; followed by a final extension at 72°C for 7 min. Ten microliters of the reaction products was analyzed on 1% agarose gel. The primer sequences are sense: 5'-GCGGAAATTCATGAGAATTTTCGAAACCACATTTGAG-3'; antisense: 5'-GCGCTCGAGAATCAATTGCAATCAAGAAGTGT-3'. The amplified cDNA was ultimately digested with *Eco*R I and *Xho* I and cloned into pcDNA3 and pET2c. The sequences of the IL-15 DNA insert were verified by sequencing with the dideoxy chain termination method (Bioasia Co., Shanghai). Sequences were analyzed by Vector NTI Suite software package (InfoMax).

Expression of recombinant protein

Escherichia coli strain BL21(DE3) (Novagen) was transformed by the pET2c-IL-15 plasmid. The detailed protocol for production of fusion proteins was as follows. *E. coli* cells were grown to absorbance 0.6 at 600 nm, then expression was induced with 0.4 mmol/L isopropyl-D-thiogalac-toside for 4 h at 37°C. The bacterial cells were harvested, the cell pellet was suspended in 50 mL MTPBS lysis buffer (150 mmol/L NaCl, 16 mmol/L NaH₂PO₄, 4 mmol/L Na₂HPO₄, pH 7.3, 1% TritonX-100) per liter of culture volume. The cells were sonicated three times, 10 s each time (Sinifer cell disrupter B-30; Branson Sonic Power), and centrifuged at 15 000 g for 10 min at 4°C. The supernatant was collected for SDS-PAGE and Western blotting.

Western blotting

E. coli cells were cultured for an indicated period of time, lysed by adding 2X Laemmli SDS sample buffer and boiled for 5 min in a water bath. The proteins were resolved by 15% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Immun-blot PVDF; Bio-Rad). Non-specific binding sites were blocked by incubating the blot in blocking buffer (5% nonfat dry milk in wash buffer) for 1 h at room temperature. The blot was washed three times (5 min each time) with wash buffer (0.5% Tween 20, 200 mmol/L NaCl, and 50 mmol/L Tris, pH 7.5), and incubated with the appropriate primary Ab overnight at 4°C. The blot was then washed three times (5 min each time) and incubated with the corresponding secondary Ab-HRP conjugate for 1 h at room temperature. The blot was washed three times again (5 min each time), and proteins were detected.

CTLL-2 proliferation assay

For CTLL-2 bioassays, COS cells were transfected with 30 µg of IL-15 expression constructs following a standard calcium-phosphate-mediated transfection protocol^[7]. For these studies roughly $2-3 \times 10^5$ adherent COS cells/well were transfected in six-well 35-mm plates. After 24 h, the medium was replaced with fresh complete DMEM, supernatants were harvested 72 h after the transfection for assay.

Aliquots (100 µL) of the supernatant and serial two-

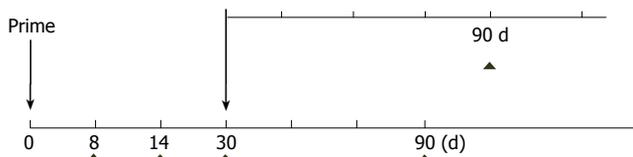


Figure 1 Immunization schedule of pHBc144 and pIL-15 plasmid.

fold dilution were added to 96-well microtiter plates containing 1×10^5 CTLL-2 cells in a final volume of 200 μ L. The cultures were incubated for 18 h at 37°C and pulse-labeled for another 6 h with 0.5 μ Ci [3 H] thymidine. Thymidine incorporation was quantified by liquid scintillation spectrometry^[8] (BD). A standard curve was plotted from proliferation induced by standard murine rIL-2.

Immunization and sample collection

Plasmids were amplified in *Escherichia coli* and plasmid DNA was purified by using anion exchange column (Endo-free plasmid purification kit, Qiagen, Chatworth, Calif) according to the manufacturer's instructions. The plasmid DNA was dissolved in sterile physiological saline, and the final concentration was 1 μ g/mL.

C57BL/6 (H-2^b) mice were vaccinated at the age of 6-8 wk by injection of DNA in 0.9% saline (w/v), into the quadriceps of each hind limb as previously described^[9]. Injection volume was 50 μ L per leg and total DNA dose was 50 μ g per leg. On d 30, all mice were booster-immunized with the same DNA. On d 0, 8, 14, 30, 90 after priming and d 90 after boosting, blood samples were collected from each mouse by orbital bleeding (Figure 1). Serum was separated from each blood sample and used for measurement of the anti-HBcAb titer. On the other hand, mouse blood was obtained using heparin as an anticoagulant. The blood was diluted with RPMI 1640. PBMCs were obtained by density gradient centrifugation over HIS-TOPAQUE-1083 (Sigma), washed three times with RPMI 1640 and counted afterward. The spleens were removed for the measurement of cytokine production.

Detection of anti-HBcIgG and subclasses

Anti-HBc antibody was titrated with ELISA kit (Shanghai Infectious Diseases' Hospital). Each serum sample was made in a series of three-fold dilution. Goat anti-mouse IgG-HRP conjugate (The Binding Site Limited Co, England) was used as the secondary antibody and O-phenylenediamine (OPD) was used as a substrate. The plates were read with Titerec Multiskan MCC/340 at A_{450} . The wells with an absorbance of ≥ 0.1 (above background, 0.020) were scored as positive.

Intracellular cytokine staining and flow cytometric (FCM) analysis

For intracellular cytokine detection of IFN- γ , PBMCs and single suspension of splenocytes were plated in a 96-well plate (Costar) at a density of 1×10^6 cells/well in a final volume of 200 μ L. Cells were stimulated with peptides for 5 h. Brefeldin A (final concentration 2 μ g/mL) was added to the cultures during stimulation. At the end of the

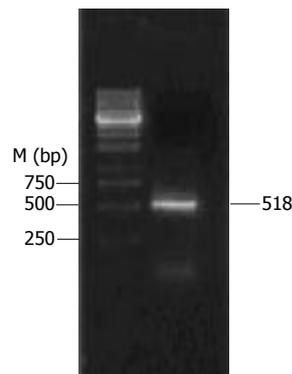


Figure 2 Electrophoretogram of PCR products. The human IL-15 coding sequence (GenBank U14407) was RT-PCR amplified from human PBL mRNA stimulated with LPS.

incubation, the cells were washed twice and stained for 30 min at 4°C with phycoerythrin (PE)-conjugated anti-mouse CD8 (BD Bioscience) in FACS buffer [2% FCS, 0.1% sodium azide in phosphate buffered saline (PBS)]. Cells were collected, washed and incubated in permeabilization buffer for 20 min on ice. The permeabilized cells were washed and stained with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IFN- γ (BD PharMingen, San Diego, CA) for 30 min. Cells were washed, fixed with 2% (w/v) paraformaldehyde, and acquired with a FACSCalibur flow cytometer (Becton Dickinson, Boston, MA). Forward and side scatters were collected on a linear scale, while the PE and FITC signals were collected on a 4-decade log scale. Overlaps of emission spectra were electronically compensated. The debris were eliminated using the threshold on forward scatter and 100 000 events were acquired with CellQuest software (BD, Biosciences).

Statistical analysis

Statistical analyses of data were performed using SPSS 10.0 for windows (SPSS, Inc., Chicago, IL).

RESULTS

cDNA cloning

PBMCs were isolated from peripheral blood of healthy volunteers by density gradient centrifugation over Ficoll. A total of 5×10^5 PBMCs were transferred into 24-well plates and cultured for 6 h with 5 μ g/mL LPS. RNA extraction and RT-PCR were performed (Figure 2). The complete human IL-15 cDNA (GenBank accession No. U14407) was 518 bp long and was comprised of 146 bp signal peptide (IL-15sp) and a IL-15 mature peptide gene sequence (IL-15mp).

Amplified cDNA was ultimately digested with *Eco*R I and *Xbo* I and cloned into pCDNA3. The sequences of IL-15 DNA insert were confirmed and this plasmid was named pIL-15.

For generation of the IL-15 prokaryotic expression construct, the IL-15 mp coding sequence was PCR amplified from pIL-15. The resulting fragments were digested with *Eco*R I and *Xbo* I and cloned into pET2c named pET-IL-15 (data not shown).

Expression of IL-15 in *Escherichia coli*

Escherichia coli strain BL21(DE3) (Novagen) was transformed by the pET-IL-15 plasmid. After induction by

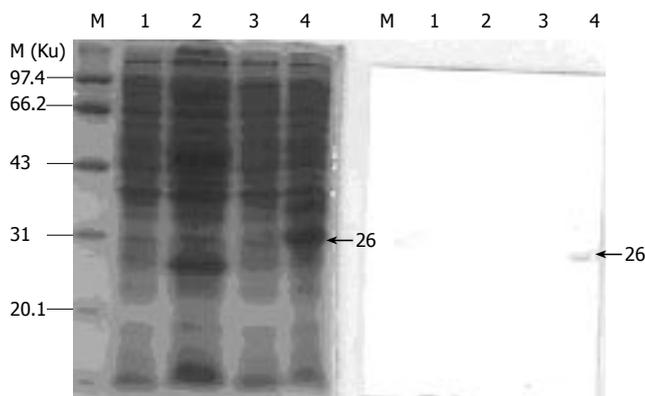


Figure 3 SDS-PAGE and Western blotting of recombinant polypeptides. M: polypeptide molecular marker; lane 1: BL21; lane 2: pET32c; lane 3: pET-15 uninduced; lane 4: pET-15 induced.

IPTG and temperature, the supernatant of bacteria lysates was taken for SDS-PAGE and Western blotting. IL-15 fragments could be expressed in pET-32c vector and its molecular weight was M_r 26 000 (Figure 3).

Production of IL-15 by COS cells

The IL-15 was cloned into the pcDNA3 vector with CMV promoter (Invitrogen) and transfected into COS cells. Forty-eight hours after transfection, the COS supernatants were collected and subjected to CTLL-2 proliferation assay. CTLL-2 cell ^3H -TdR cpm was related to the dilution fold of the COS supernatant. When the dilution fold of transfection supernatant increased, the incorporation of ^3H -TdR cpm decreased. The incorporation ^3H -TdR cpm of pIL-15-transfected COS supernatant was higher than that of vector-transfected COS supernatant (Figure 4). So we could detect IL-15 activity in the supernatants of COS cells transfected with pIL-15.

Kinetics of antigen-specific CD8 responses following DNA vaccination and cytokine expression profiles of antigen-specific T cells

Both primary and memory antigen-experienced CD8⁺ T cells rapidly upregulated cytokine production after encountering cells displaying their cognate peptide ligand in association with a major histocompatibility complex class I molecule. We used ICCS to identify cytokine-producing HBc-specific CD8⁺ memory T cells directly *ex vivo* after DNA inoculation prior to this study, but it was unclear how quickly these responses developed when IL-15 as a gene adjuvant was coinjected with HBc DNA vaccine. Therefore, we used ICCS to directly enumerate antigen-specific effector CD8⁺ T cells in the spleens of mice at various time points after DNA immunization (pHBc144 and/or pIL-15). The average percentage of peptide-specific CD8⁺ T cells detected in three mice analyzed at each time point for each mode of immunization is shown in Figure 5A.

Seven days after-immunization, antigen specific CD8⁺ T cells were present at a frequency of less than 0.1% in all of the vaccinated mice (both pHBc144 + pcDNA3 group and pHBc144 + pIL-15 groups), and therefore accurate

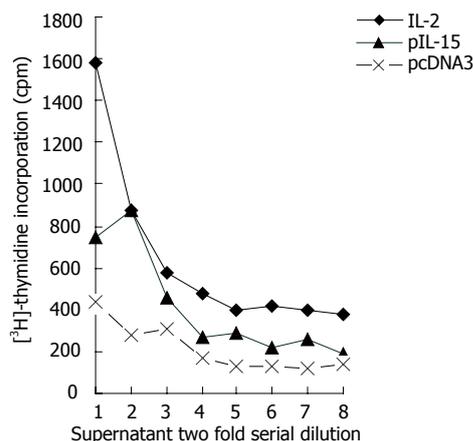


Figure 4 IL-15 bioactivity in supernatants of cells transfected with pIL-15 expression plasmid. COS cells were transfected with pIL-15 (\blacktriangle) expression constructs or vector pcDNA3 (\times), and the supernatants were collected 72 h after transfection. The supernatants were tested by CTLL-2 bioassay to measure IL-15 bioactivity. IL-2 (5×10^3 U/mL) was used as a positive control.

analysis of IFN- γ production was not possible (Figure 5A). By d 14, $2.64\% \pm 1.07\%$ of the HBcAg specific CD8⁺ T cells primed by pHBc144 + pIL-15 DNA vaccination secreted IFN- γ , and at 30 d after-immunization, $0.39\% \pm 0.21\%$ of the antigen-specific cells were positive. A similar transition was observed in CD8⁺ T cells primed by pHBc144 + pcDNA3 DNA vaccination ($2.26\% \pm 2.50\%$ on d 14 and $0.42\% \pm 0.06\%$ on d 30). Thus, the cytokine expression patterns of HBcAg-specific T cells were similar regardless of the pIL-15 immunization.

These proportions decreased gradually, by d 90 post-priming, $0.48\% \pm 0.36\%$ of the antigen-specific CD8⁺ T cells induced by pHBc144 in pIL-15 co-immunized group secreted IFN- γ , while $0.16\% \pm 0.04\%$ of the antigen-specific CD8⁺ T cells in pHBc144 and pcDNA3 co-immunized group were IFN- γ ⁺ CD8⁺ T cells (Figure 6).

After being boosted, the HBc Ag specific T cells expanded rapidly, and by d 90 post-boost the specific memory cells could still be detected, sustaining a higher level than that for the first response. On d 90 after boosting, $0.28\% \pm 0.04\%$ of the antigen-specific CD8⁺ T cells induced by pHBc144 in pIL-15 co-immunized group secreted IFN- γ , while $0.11\% \pm 0.04\%$ of the antigen-specific CD8⁺ T cells in pHBc144 and pcDNA3 co-immunized group were IFN- γ ⁺ CD8⁺ T cells (Figures 6A and B).

Kinetics of HBcAg specific CD8⁺ T-cells in the circulation induced by DNA vaccine

For examining the kinetics and distribution of antigen specific CD8⁺ T-cells in the non-lymphoid tissue induced by DNA vaccination, we separated the lymphocytes from blood. The kinetics of HBcAg-specific CD8⁺ T cells in the circulation stimulated with pHBc144 showed a similar pattern with that of the spleen (Figure 5). However the number of HBcAg-specific CD8⁺ T cells in PBMCs was lower than that in the spleen at the same time point. Eight days after DNA vaccination, HBcAg-specific CD8⁺ T cells were detectable. By d 14 the number of IFN- γ ⁺ CD8⁺ T cells in PBMCs reached the peak and then dropped gradu-

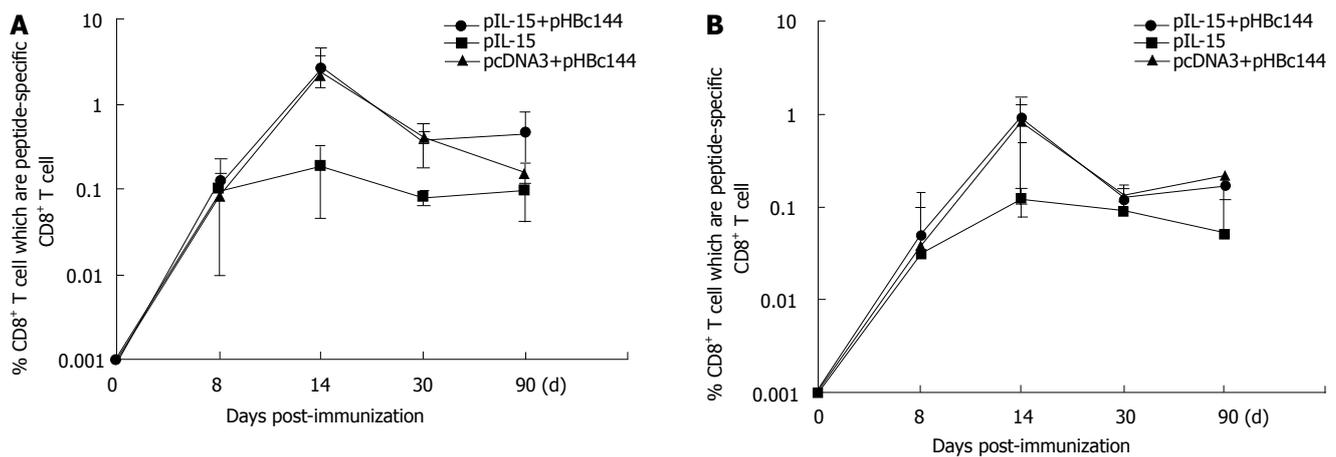


Figure 5 Kinetics of antigen-specific CD8⁺ T-cell response in spleen (A) and PBMCs (B) following DNA immunization. Mice were immunized with pHBc144 plasmid DNA or vector DNA intramuscularly. At various time points, splenocytes and PBMCs were harvested, stimulated for 5 h with specific peptide, double stained with anti-CD8a-PE and anti-IFN- γ -APC. A total of 1×10^5 events were acquired by a flow cytometer. The indicated values on the lines of the figure (plotted on a log₁₀ scale) reflect the percentage of peptide-specific CD8⁺ T-cells to total CD8⁺ T-cells in the spleens or PBMCs (about three mice/group at each time point). The cut-off value was 0.1%.

Table 1 Total CD4⁺ and CD8⁺ T-cells detected in spleen and PBMC from mice co-immunized with pHBc144 and pIL-15 following DNA immunization

A Spleen

Group	d 8		d 14		d 30		d 90		booster d 90	
	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8
pIL-15+pHBc144	13.35 ± 1.00	8.31 ± 2.08	17.89 ± 0.83	15.15 ± 0.88	22.02 ± 2.12	12.63 ± 0.51	20.23 ± 3.07	23.41 ± 0.65	11.76 ± 2.36	12.10 ± 0.59
pcDNA3+pHBc144	14.08 ± 1.32	8.24 ± 1.08	16.69 ± 2.21	14.75 ± 0.87	19.56 ± 1.23	14.58 ± 0.98	19.77 ± 3.61	22.37 ± 1.53	12.03 ± 2.50	13.43 ± 1.93
pIL-15	14.43	10.12	14.69 ± 2.25	10.31 ± 1.94	19.76 ± 1.41	13.38 ± 1.53	19.04 ± 1.75	15.72 ± 3.08	11.08 ± 0.21	12.76 ± 2.55

B PBMC

Group	d 8		d 14		d 30		d 90		booster d 90	
	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8
pIL-15+pHBc144	13.05 ± 1.75	7.42 ± 1.41	15.93 ± 2.95	10.67 ± 1.87	19.10 ± 3.70	10.06 ± 0.47	16.94 ± 1.19	8.61 ± 1.20	8.66 ± 0.13	12.78 ± 1.76
pcDNA3+pHBc144	15.49 ± 4.77	8.35 ± 1.84	18.30 ± 3.56	12.24 ± 1.27	22.97 ± 3.17	12.79 ± 4.39	7.50 ± 0.58	7.18 ± 2.93	10.09 ± 5.19	16.20 ± 1.48
pcDNA3+pHBc144	14.85	8.92	17.98 ± 3.20	7.68 ± 3.46	21.70 ± 5.44	12.51 ± 1.16	12.58 ± 0.66	9.43 ± 1.36	8.36 ± 3.53	10.34 ± 2.09

Mice were injected with 100 μ g of pIL-15 or pHBc144 or both pIL-15 and pHBc144 plasmids intramuscularly. Single-cell suspensions were prepared. Cells were stained with anti-CD4-APC and anti-CD8-PE. The stained cells were analyzed with a FACScan. The data from each group at each time point represent the mean \pm SD ($n = 3$). (A) the total CD4 and CD8 in the spleen. (B) the total CD4 and CD8 in the PBMC.

ally. These proportions decreased gradually until d 90 post-priming, 0.17% \pm 0.05% of the antigen-specific CD8⁺ T cells induced by pHBc144 in pIL-15 co-immunized group secreted IFN- γ , while 0.22% \pm 0.01% antigen-specific CD8⁺ T cells in pHBc144 and pcDNA3 co-immunized group were IFN- γ ⁺ CD8⁺ T cells.

On d 90, after boosting, 0.20% \pm 0.13% of the antigen-specific CD8⁺ T cells induced by pHBc144 and pIL-15 co-immunized group secreted IFN- γ , while 0.12% \pm 0.13% antigen-specific CD8⁺ T cells in pHBc144 and pcDNA3 co-immunized group were IFN- γ ⁺ CD8⁺ T cells (Figure 5B, Figure 6 C and D). The antigen-specific memory cells still could be detected directly *ex vivo* in the circulation on d 90 post-boost (Figure 5B, Figure 6D).

HBcAg specific CD4/CD8 in spleen and PBMCs induced by HBcDNA vaccine

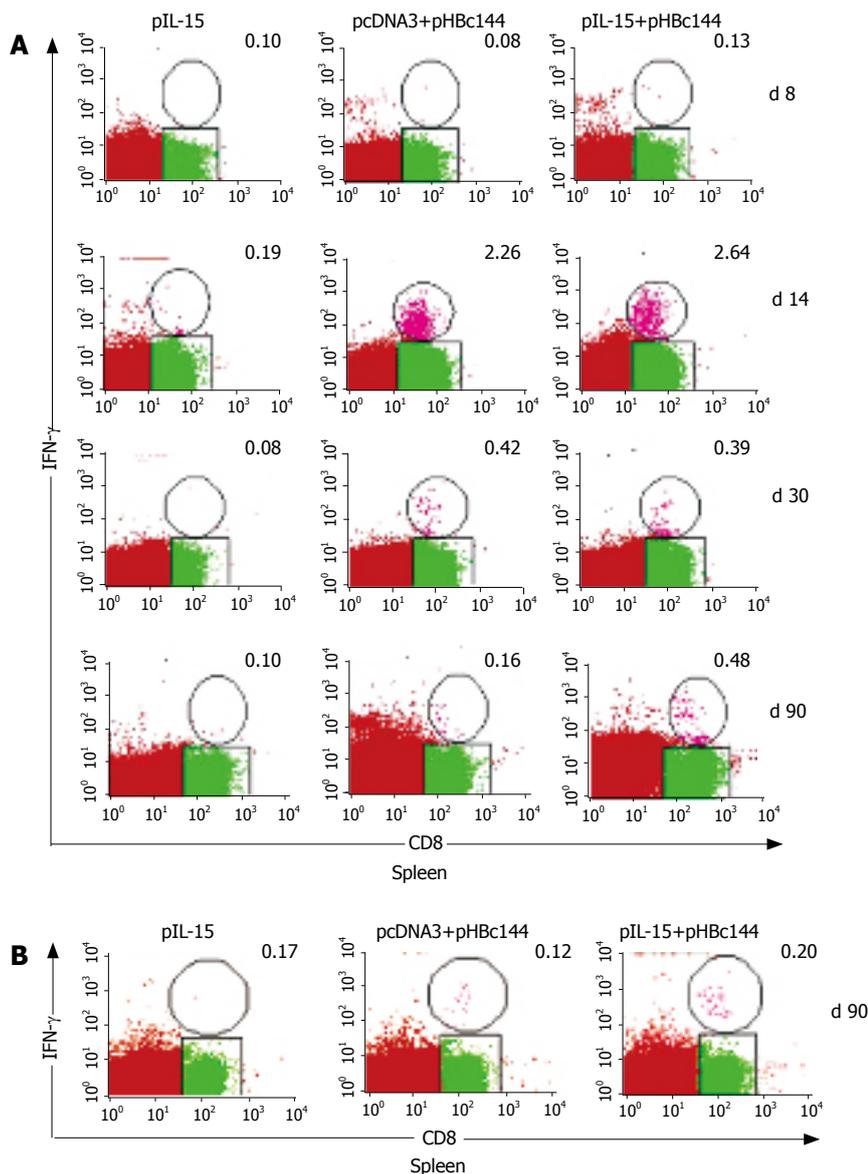
At the same time point, we checked the total CD4⁺ and

CD8⁺ cell percentage (Table 1), IL-15 had no big effect on the ratio of CD4⁺ and CD8⁺.

HBcAg specific humoral response induced by DNA vaccine

The HBcAg specific humoral immune response induced by pHBc144 was detected by ELISA for anti-HBc antibody in serum. The plates were read with Titerek Multiskan MCC/340 at A_{450} . The wells with an absorbance of ≥ 0.1 (above background, 0.020) were scored as positive.

Four weeks after the first pHBc144 immunization, all mice underwent anti-HBc seroconversion. The mean titer of anti-HBc IgG in pHBc144 + pIL-15 group reached $2^{9.81} \pm 2^{0.9}$ on d 30. After being boosted, the mice produced a higher titer of anti-HBc IgG ($2^{9.81} \pm 2^{0.9}$) than that of the primary response, and anti-HBc maintained at that level ($2^{9.81} \pm 2^{0.9}$) on d 90. The titer of anti-HBc IgG2a in pHBc144 + pIL-15 group ($2^{9.81} \pm 2^{0.9}$) was slightly higher



than that of pHBc144 + pcDNA3 group ($2^{8.22} \pm 2^{0.9}$), but there was no significant difference ($P > 0.05$).

DISCUSSION

IL-15 plays a pivotal role in the development, survival, and activation of NK cells, NK-T cells and cytokine release various subsets of T and B cells^[10-12]. IL-15 maintains the homeostasis of memory phenotype CD8⁺ T cells^[13]. The IL-15 response to infectious agents, such as viruses and other intracellular organisms, may represent a critical element in the host defense against these pathogens. Optimized IL-15 in combin with HIV-1gag DNA constructs results in a significant enhancement of Ag-specific CD8⁺ T cell proliferation and IFN-gamma secretion, and strong induction of long-lived CD8⁺ T cell responses^[14]. pDNA encoding IL-15 is capable of elevating survival rates of HSV-1-infected mice when coinjected with 1 μ g of gB pDNA^[15]. IL-15 has been reported to have a profound effect on the augmentation of CD8⁺ T cell response against murine *T. gondii* infection^[16]. *Toxoplasma*-specific CD8⁺ T cell immunity in mice is depleted over time, but can be rescued by IL-15 treatment. These findings suggest that IL-15

may be useful as an immune adjuvant given with vaccination to enhance its biologic efficacy.

IL-15 is predominantly regulated posttranscriptionally at the level of translation and translocation. Although Northern blot analysis can reveal widespread constitutive expression of IL-15 mRNA in a variety of tissues such as placenta, skeletal muscle, kidney, lung, heart, fibroblasts, epithelial cells, and monocytes, it is difficult to demonstrate IL-15 in supernatants of many cells that express such mRNA. This discordance between IL-15 mRNA expression and IL-15 protein production is regulated by multiple elements including 12 upstream AUGs of the 50 UTR, a 48-aa signal peptide, and the C-terminus of the mature protein. The IL-15 signal peptide is an important factor in the negative regulation of IL-15 protein expression. There are two alternative leader peptides, one with 48 aa and the other with 21 aa^[17]. IL-15 associated with a short 21-aa signal peptide is not secreted but stored intracellularly, appearing in nuclear and cytoplasmic components^[18]. The long 48-aa isoform of the signal peptide might function as a negative regulator of IL-15 generation. The total quantity of IL-15 generated (the sum of IL-15 retained within the cells) increases 17- to 20-fold when the IL-15

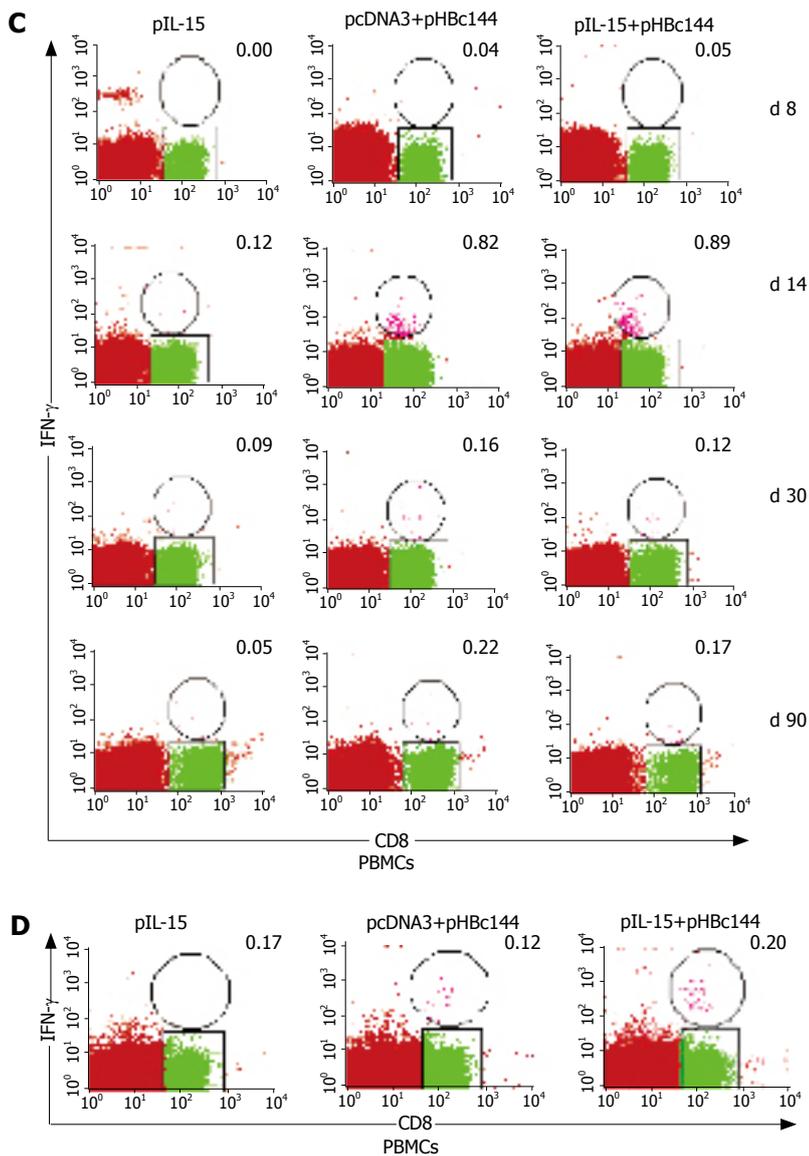


Figure 6 Percentages of Hbc specific CD8⁺ T cells in the spleen after primary (A) and secondary immunization (B) and in PBMCs after primary (C) and secondary immunization (D) detected by ICCs directly ex vivo at various time points. C57BL/6 mice were immunized with pHBc144 DNA or pIL-15 DNA and boosted at d 30 later. The splenocytes and harvested at various time points. Then the cells were cultured for 5 h in the absence or presence of the HBcAg peptide and double stained with anti-CD8a-PE and anti-IFN- γ -APC. CD8⁺IFN- γ ⁺ cells are enclosed in ovals, and the numbers in the upper right quadrants represent the percentage of CD8⁺IFN- γ ⁺ cells to total CD8⁺ T cells. The data from each group at each time point represent mean \pm SD ($n = 3$). Naive mice were used as negative controls. The cut-off value was 0.1%.

signal peptide is replaced by that of IL-2. Parallel studies showed that the quantity of IL-2 secreted is reduced 40- to 50-fold when COS cells are transfected with the reciprocal construct in which the IL-2 signal peptide is replaced with that of IL-15. Furthermore, after the IL-15 signal peptide is replaced with that of CD33^[19], translation and secretion increase, supporting the view that IL-15 expression is controlled posttranscriptionally at the level of translation and secretion.

We cloned the classical 48-aa signal peptide isoform of IL-15 (LSP-IL-15). When COS cells were transfected with the wild-type construct^[19], the quantity of IL-15 protein was very low (360 pg per 200 000 cells). Moreover, in most cases it was difficult to demonstrate IL-15 in the culture supernatants. We could not detect IL-15 protein in culture supernatants by Western blot after transfecting COS cells with our construct (data not shown). We therefore sub-

cloned the LSP-IL-15 cDNA into expression vector and let it translate in *E. coli*. Western blot analysis showed a Mr 26 000 fusion protein, which was identified with NCBI sequence.

The IL-15 associated with the long 48-aa signal peptide enters the endoplasmic reticulum where it was glycosylated. Nevertheless, IL-15 is secreted after trafficking through the Golgi, yielding a cytokine with a fully processed signal peptide. We measured the biological function of the construct by detecting the IL-15 in the supernatant of transfection.

Meazza *et al.*^[20] and Onu *et al.*^[17] found that IL-15 is not secreted when IL-15 SP is employed. Nevertheless, Waldmann *et al.*^[21] assessed cultures from COS cells transfected with the wild-type IL-15 coding sequence at 120 h and found that at least 90% of the IL-15 protein (though at low levels) can be identified in the culture

supernatants, and 10% is retained within the cells, as evaluated by sensitive ELISA assays. Our result is consistent with that of Waldmann *et al.* Although in COS cells transfected with IL-15 expression construct, the levels of IL-15 protein synthesized and secreted were very low (3 logs less than those obtained with a comparable IL-2 construct), LSP-IL-15 could secrete in the supernatant of COS cells transfected with IL-15 expression construct and have bioactivity, and can induce CTLL-2 cell proliferation. This difference between the different studies may be due to the fact that the CTLL-2 strain cannot respond to IL-15.

To determine if the IL-15 plasmid has many effects on the humoral and cellular immune response, especially CD8⁺ T cell response induced by DNA vaccination, splenocytes and PBMCs from the pIL-15 and HBcAg DNA vaccine co-immunized mice were evaluated for IFN- γ expression on antigen-specific CD8⁺ T cells and anti-HBc IgG in serum was detected at each time point. We selected them for the DNA plasmid format because they are inexpensive, noninfectious and can be readily optimized to enhance immunogenicity and protective efficacy. Furthermore, DNA vaccines can induce robust and durable CD8⁺ T cell responses, and can be used to deliver a large number of CTL epitopes^[22]. Our results showed that pIL-15 had no effect on anti-HBc IgG induced by pHbc144. Although antigen-specific CD8⁺ T cells reached a peak on d 14 after priming, pIL-15 had no effect on antigen-specific CD8⁺ T cells in splenocytes or PBMCs induced by HBcAg DNA vaccine. pIL-15 could increase the percentage of antigen-specific CD8⁺ T cells induced by HBcAg DNA vaccine on d 90 after priming and boosting. These two time points belonged to memory phase of immune response.

The existence of the basic effector Th1 and Th2 subsets is now well accepted, and used to plan therapeutic and vaccine strategies. Th1 cells produce interleukin 2 (IL-2), interferon γ and lymphotoxin (LT), whereas Th2 cells produce IL-4, IL-5, IL-6, IL-9 and IL-13^[23]. The function of Th1 and Th2 cells correlates well with their distinctive cytokines. Th1 cells are involved in cell-mediated inflammatory reactions. Several Th1 cytokines activate cytotoxic and inflammatory functions, while Th1 clones induce delayed-type hypersensitivity (DTH) reactions; and IFN- γ is commonly expressed at sites of DTH reactions Th2 cytokines promote antibody production, particularly IgE responses, and also enhance eosinophil proliferation and function. Accordingly, Th2 cytokines are commonly found in association with strong antibody and allergic responses. Unfortunately, we did not evaluate the effect of IL-15 on antigen specific Th1 cells induced by HBc DNA vaccine. But our results showed that IL-15 had no effect on the ratio of CD4⁺ and CD8⁺.

Chronic HBV infection is one of the most common infectious diseases worldwide and leads to a high morbidity and mortality due to the development of liver cirrhosis and cancer. Virus persistence is believed to be due to an insufficient antiviral Th cell and CTL response of the host^[24]. Effective HBV-specific CD8⁺ T cell responses have been proven to inhibit virus replication independently of liver damage^[25,26].

During HBV replication, the nucleocapsid or core particle recruits into the pregenomic RNA and viral reverse transcriptase, and provides the template on which the surface envelope components assemble. Recombinant Mr 21 000 (P21) core protein self-assembles into 28-nm particles representing the native hepatitis B core Ag (HBc Ag)^[27]. Since it plays a central role in nucleocapsid formation and pregenomic viral RNA packaging, HBV core antigen represents another interesting target for DNA-based vaccine, although less data are available on DNA immunization to core antigen protein compared to the envelope protein. DNA-based immunization of mice to the HBV core has been shown to efficiently prime specific antibody and cytotoxic-T-lymphocyte responses^[28].

Interferon is one of the most effective drugs for chronic HBV infection^[29]. Experiments with HBV transgenic mice suggest that intrahepatic secretion of antiviral cytokines, such as IFN- γ and TNF- α by CTLs and Kupffer cells can interrupt the HBV life cycle without lysis of infected hepatocytes. The rapid reduction of HBV-DNA before hepatocellular damage in acute infections in chimpanzees and humans suggests that a similar non-cytolytic mechanism is, at least in part, responsible for inhibition of viral replication during natural infection^[30].

In conclusion, DNA vaccine constructed by HBc Ag 1-144 amino acid induces effective cell immunity, cytokine plasmid-delivered IL-15 enhances CD8⁺ T cell longevity, IL-15 is a possible candidate adjuvant for HBV DNA vaccine.

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RAPID COMMUNICATION

Hand-assisted laparoscopic surgery of abdominal large visceral organs

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Abstract

AIM: To design a hand-assisted laparoscopic approach in an attempt to provide an option for laparoscopic resection of abdominal large viscera.

METHODS: A 5-6 cm incision (for HandPort) and 2 trocars were employed. The main vessel of the target organ was taken as a "core", and all tissues around the core were taken as peripheral structures. The peripheral structures were dissected first, and the core vessels were treated last. Twenty-six patients underwent laparoscopic deroofting of the hepatic huge cysts, resection of the segments lying at the outer edge of the liver (segments 2 to 6), splenectomy, hemicolectomy, ileocectomy and subtotal gastrectomy with HandPort device, harmonic scalpel, or Ligasure.

RESULTS: The duration of the procedure was within 2 hours. Blood loss amounted to 8-120 mL. The conversion rate was 3.8% (1/26). All patients had uneventful postoperative courses with less pain, earlier oral intake, and faster recovery, compared with conventional surgery.

CONCLUSION: This method combines the advantages of both open and laparoscopic techniques, achieving better hemostasis effect, shortening the operative time, and is beneficial to the patients.

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Key words: Minimally invasive surgery; Hand-assisted laparoscopic surgery; Hepatic segmentectomy; Hemicolectomy; Subtotal gastrectomy

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INTRODUCTION

Since Mouret performed the successful laparoscopic cholecystectomy in 1987, new frontiers have opened for minimally invasive surgery^[1], and has been expanded to more extensive procedures such as resection of the abdominal large viscera^[2-4]. The minimally invasive surgery is superior to conventional open surgery because it offers significant benefits to the patients including much shorter incision, less pain and faster recovery. Despite instrumentation has dramatically improved over the past decade, laparoscopic resection of abdominal large organ still has several concerns regarding the safety, technical difficulties, rapidity and complications such as bleeding, injury to major adjacent structures, which affect the short-term and long-term outcomes^[5].

In an effort to largely simplify and shorten the procedure, provide an easier, safer approach, and decrease patients' postoperative pain and stay, we have designed a method for laparoscopic resection of abdominal large viscera. Usually, several 5-12 mm trocars are placed in the abdominal wall in laparoscopic surgery. However, there must be 5-6 cm port for extraction of a resected specimen in removal of abdominal large visceral organ. From "1 port + 2 trocars" as the minimal invasive wounds, we used a quick and safe hand-assisted laparoscopic technique for resection of abdominal large viscera, by taking advantages of both open and laparoscopic techniques.

MATERIALS AND METHODS

Patients

From May 2001 to December 2002, 26 patients, 15 males and 11 females with a mean age of 61.8 years (range 42-71 years), underwent hand-assisted laparoscopic surgery involving resection of liver, spleen, colon, jejunum, ileocecum, stomach and gallbladder, including 2 emergency operations: a 71 year-old female had uncontrolled bleeding from multiple diverticulosis of ascending colon, and another 54 year-old female suffered from carcinoma of terminal ileum associated with ileus. The procedure was carried out endocorporeally and extracorporeally, by means of the HandPort system (Smith & Nephew Inc. Andover, Massachusetts), harmonic scalpel (HCS 15 and HCS C5, Ethicon, Endo-surgery, Cincinnati, Ohio. Ultra shears 5 mm instrument, US Surgical, Tyco Healthcare) or Ligasure (10 mm Atlas, Vallylab, Boulder, Tyco Healthcare). All procedures were performed with the patients in the

Table 1 (HALS) operations and indicating diseases (26 cases)

Cases	Operations	Indicating diseases
1-2	Left lateral segmentectomy	Hepatitis B-related cirrhosis supervened hepatocellular carcinoma (HCC), hepatic cavernous hemangioma
3	S 6 segmentectomy	Hepatitis B-related cirrhosis supervened HCC
4	S 5 and 6 segmentectomy	Hepatitis B-related cirrhosis supervened HCC
5	S 5, 6 and partial 4 segmentectomy + cholecystectomy	Hepatitis B-related cirrhosis supervened HCC
6	Partial S 4 segmentectomy	Hepatic cavernous hemangioma
7-11	Deroofing of the hepatic huge cysts	The 4, 6, 7, 8 segmental hepatic cysts; the 4, 5, 6, 7, 8 segmental hepatic cysts; the 5, 6, 7 segmental hepatic cysts (2); the 5, 6, 7 segmental hepatic cysts with calcification of cystic wall
12-15	Right hemicolectomy	Carcinoma of the ascending colon (3) ¹ Multiple diverticulosis of ascending colon with massive hemorrhage
16	Left hemicolectomy + partial jejunectomy	Carcinoma of the splenic flexure of colon with metastasizing to liver and jejunum,
17	Left hemicolectomy	² Carcinoma of the sigmoid with metastasizing to left ureter and common iliac artery
18-19	Ileocectomy	¹ Carcinoma of the terminal ileum with ileus, multiple diverticulosis of the ileocecum and chronic appendicitis
20-21	Splenectomy	Idiopathic thrombocytopenic purpura, carcinoma of pancreatic body and tail
22-25	Subtotal gastrectomy	Duodenal ulcer (2), gastric ulcer, gastric ulcer with massive hemorrhage
26	Half gastrectomy + cholecystectomy	Diverticulitis of duodenum and gall stone

¹Emergency operation; ²Conversion to open operation.

Table 2 Location of the ports and positioning of the operator

Operations	HandPort	Camera ¹	Scalpel ¹	Operator ²
Left hepatic lateral segmentectomy	R upper transrectus ³	Umbilicus	L upper quadrant	R
Right hepatic segmentectomy	R pararectus abdominis	Umbilicus	L upper quadrant	R
Deroofing of the hepatic huge cyst	R subcosta	Umbilicus	L upper quadrant	L
	R upper transrectus	Umbilicus	L upper quadrant	L
	R pararectus abdominis	Umbilicus	L upper quadrant	R
Splenectomy	Upper midline ⁴	Umbilicus	L umbilicus level	R
	L upper transrectus	Umbilicus	L lower umbilicus level	R
Right hemicolectomy	Upper midline	Umbilicus	Lower midline	R
Left hemicolectomy + partial jejunectomy	Upper midline	Umbilicus	Lower midline	R
Subtotal gastrectomy	Upper midline	Umbilicus	L lower umbilicus level	R
Half gastrectomy + cholecystectomy	Upper midline	Umbilicus	R lower umbilicus level	R
Ileocectomy	Lower midline ³	Umbilicus	R middle quadrant	L

¹Camera and Scalpel can change places with each other during operation; ²Left hand into abdomen, operator can change places during operation; ³Two incisions for HandPort device were at the previous wounds of right upper transrectus abdominis and lower midline involved open partial gastrectomy and caesarean operation, respectively; ⁴Just under the xiphoid process; R: right, L: left.

supine position, rotated appropriately, or with rolls under the flank of the appropriate side. The operations and indicating diseases are listed in Table 1.

The location of HandPort device was preferred to be directly over or close to the main (core) vessels of the target organ, in order to provide direct visualization to transect the core vessels or dissect the radical lymph nodes. The location of ports and positioning of the operator are listed in Table 2.

Before placement of HandPort device

A 5-6 cm abdominal incision was performed, before placement of HandPort device to allow for introduction of the hand to assist in placing two trocars, similar to placement of abdominal drains in open surgery, and also to allow the hands to perform abdominal exploration. If the dissection preceded the resection, the following procedures were done under direct vision, including mobilizing intraperitoneal adhesion, dissecting the peripheral structures, or placing a loop for occlusion of

hepatic hilar pedicles, in case of emergency use.

Following placement of HandPort device

The HandPort device was placed, with the abdomen insufflated under a pressure of 13-14 mmHg. The left hand was reintroduced. While exposing and retracting or pulling tissues with the hand, the peripheral structures including adhesions, peritoneum, ligaments, omentum, mesentery, as well as the hepatic parenchyma around the "core" pedicles were dissected by harmonic scalpel or Ligasure, occasionally by combined use of electrocoagulation without any laparoscopic suture or ligation.

Treatment of the core vessels finally

Once the target organ was mobilized, its core vessels were treated openly through the port incision by conventional open technique or vascular endo-stapler, as the location of the port incision was most direct over or close to the core vessels of the target organ.

Among our patients, a 71 year-old woman suffered

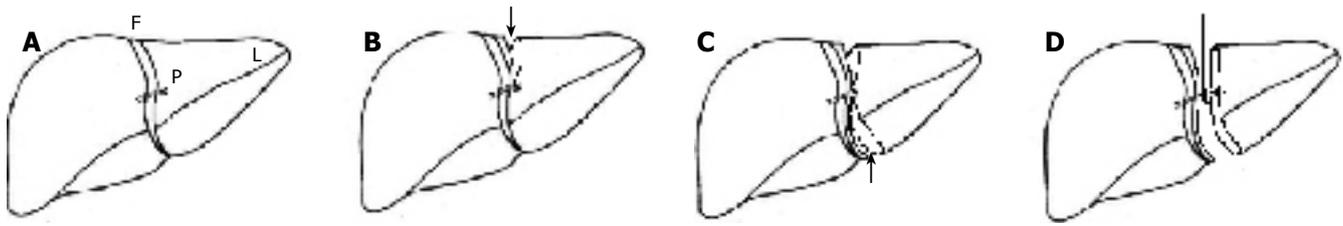


Figure 1 A: F, falciform ligament; P, portal pedicles; L, lateral segment; B: Dissecting from top to bottom till down to core vessels; C: Dissecting from bottom to top till up to core vessels; D: Transecting core vessels openly using an endo-stapler.

Table 3 Indications for conventional surgery (25 cases)

Cases	Operations	Indications
1-2	Left lateral segmentectomy	Hepatitis B-related cirrhosis supervened HCC, hepatic cavernous hemangioma
3-6	Deroofing of the hepatic huge cysts	Right hepatic huge cysts (4)
7-8	Hepatic S 5 and 6 segmentectomy	Hepatic cavernous hemangioma, hepatitis B-related cirrhosis supervened HCC
9-10	Hepatic S 6 segmentectomy	Hepatitis B-related cirrhosis supervened HCC, hepatic cavernous hemangioma
11	Hepatic S 5 segmentectomy + cholecystectomy	Hepatitis B-related cirrhosis supervened HCC
12-13	Splenectomy	Splenic infarction, splenic cysts
14-17	Right hemicolectomy	Carcinoma of the ascending colon (3), multiple polyps of ascending colon
18	Left hemicolectomy	Carcinoma of the descending colon
19-20	Ileocectomy	Carcinoma of the terminal ileum, multiple diverticulosis
21-25	Subtotal gastrectomy	Duodenal ulcer(2), ¹ gastric ulcer with bleeding, gastric ulcer, diverticulitis of duodenum with bleeding

¹Emergency operation.

Table 4 HALS compared with conventional open surgery

	Cases	Incision (cm)	Time of operation (min)	Operative bleeding (mL)	Postoperative feeding (d)	Hospital stay (d)
Open surgery	25	16-33	125-240	80-900	2-6	7-26
(mean)		22.5	190	340	4	13
HALS	25 ¹	5-6	30-120	8-120	1-3	4-15
(mean)		5.3	86	85	1.8	7

¹One of the 26 patients converting to open operation was not included. Statistical comparison was performed using the Independent-Samples *t*-Test ($P < 0.001$).

from hepatitis B-related cirrhosis supervened HCC and arrhythmia. She had had an open partial gastrectomy 5 years ago due to leiomyoma of stomach. She underwent hand-assisted laparoscopic left lateral segmentectomy. The HandPort incision of 6 cm in length was located at the previous wound scar at the right upper transrectus abdominis (the previous incision of 18 cm in length). The strategy of the procedure was to treat the portal pedicles of the left lateral segment as core vessels. First, the adhesions between wound peritoneum and omentum, liver and stomach were dissected. A loop was then placed around hepatic hilar pedicles, in case of requiring interrupting hepatic afferent blood flow. Two trocars for camera and harmonic scalpel were introduced by the open method of making abdominal drains. Following placement of HandPort system, the left hand was reintroduced and the target segment retracted. Ligament attachments of the left lobe were divided until the left lateral segment was completely freed, as shown in Figure 1A. The peripheral

parenchyma around the core vessels was dissected from top toward bottom down to the core vessels (portal pedicles) with a harmonic scalpel (HCS 15 from Ethicon, Cincinnati) (Figure 1B). Then, dissection switched from the bottom toward top up to the core vessels. The core vessels were completely freed (Figure 1C). Finally, the core vessels were transected by a vascular endo-stapler through the port incision openly (Figure 1D). The resected specimen was extracted immediately. There was no active blood oozing from the resected raw surface of the liver, which was covered with absorbable hemostasis gauze. The blood loss was less than 60 mL, without temporary inflow occlusion during operation. There was no need for abdominal drain. The resected left lateral segment was 15 cm × 8 cm × 4 cm.

RESULTS

Twenty-six patients underwent uneventful operation courses. One patient was converted to open procedure because his left ureter and common iliac artery were invaded by the sigmoid cancer. The conversion rate was 3.8% (1/26). The length of the procedures was under 2 hours (range 30 min-2 h). The operative blood loss was between 8 to 120 mL. There were no postoperative complications except that a 70 year-old man with chronic pulmonary emphysema and carcinoma of ascending colon, who underwent hand-assisted laparoscopic right hemicolectomy, developed 2 split stitches at the wound because of a violent cough on the 2nd postoperative day, and another patient had a wound stitch reaction. After operation, all patients felt less pain, resumed earlier oral intake of food, and faster recovery, compared with 25

consecutive corresponding cases of conventional surgery. The data are summarized in Tables 3 and 4.

DISCUSSION

Compared with full minimal laparoscopic large organ resection, an incision is often required for extraction of the resected specimen. In fact, there are often “1 port + more than 2 trocars” abdominal wounds in full minimal laparoscopic large viscera resection. By use of currently available tools including HandPort system, harmonic scalpel, Ligasure[®], we designed “1 port + 2 trocars” specific HALS for safe and quick resection of large abdominal visceral organs.

This technique can be of real significance in avoidance of vascular bleeding during removal of the target organ, because the final step is transection of the core vessels. There is no need for worrying about bleeding as the staplers are taken off. Furthermore, a hand inside the abdomen can play an important role in preventing or controlling bleeding. On the contrary, for total laparoscopic operation, which were several times higher in blood loss and longer in operative time, the unmanageable bleeding has become the main cause to convert to open surgery. Total laparoscopic operation does not equal to the minimally invasive surgery. The minimally invasive surgery presents not only the minimal abdominal wound, but also the minimal surgical intervention and complications including bleeding, injury to major adjacent structures, conversion to open surgery and operative time. The technique we employed is by all means the small ports to accomplish the big organ resection with minimal surgical stress, better hemostasis and shorter operative time, and faster recovery of the patients.

An excellent combination of laparoscopic and open techniques for hemicolectomy

Hand-assisted laparoscopic hemicolectomy by this technique is presumed to be an excellent approach combining advantages of laparoscopic and open surgeries. The procedure includes: (1) Laparoscopically, the distal peripheral structures are mobilized by harmonic scalpel or Ligasure with hand-assistance; (2) Conventionally, the mobilized target colon is long enough to be extracted through the optimal base retractor of HandPort system, through which the major core vessel pedicles are transected, or the radical lymph nodes are dissected under direct vision, then the specimen is removed and delivered to prevent port side “seeding”; (3) Extracorporeally, end to end coloileostomy or colocolostomy is performed by sutureless biofragmentable anastomosis ring (BAR), or single layer anastomosis.

In our series, the HALS wound length of 5-6 cm (mean 5.3 cm) was much shorter than the open surgical wound length of 16-33 cm (mean 22.5 cm). Other studies have reported full laparoscopic wound length of 8.5 cm^[7]. The operative time in our group was under 2 h. In addition, previous studies revealed mean operative times for full laparoscopic colectomy, converted procedures, right, and sigmoid resection were 164, 203, 121 and 177 min, respectively^[8].

Advantages over full laparoscopic splenectomy

Our specifically designed HALS in splenectomy has advantage over total laparoscopic splenectomy. First, through the small incision port, the gastrosplenic ligament was dissected as much as possible by open technique before pneumoperitoneum. Second, after placement of the HandPort device, a hand into the abdomen assisted harmonic scalpel or Ligasure to dissect the rest ligaments. It seemed the spleen was retracted by hand with more safety. It also provided better exposure, easier and faster performance than other instruments. Third, splenic pedicle was transected by endo-stapler laparoscopically, or through the small incision with open visualization. Lastly, the resected specimen was extracted immediately after removal of the spleen. However, in full laparoscopic splenectomy with about 4 trocars, the specimen is bagged, one port incision is extended, and the spleen is broken into pieces, and then delivered.

A definite advantage in terms of HALS in hepatectomy

Our study demonstrates HALS in resection of the segments lying at outer edge of the liver (segments 2 to 6) can be performed with ease, safety and rapidity. Placement of the hand into the abdomen plays an important role, because there is no ideal instrument available for grasping the liver but the hand, which acts as a comparatively atraumatic grasping clamp and retractor providing good exposure, and also allows for fingers to identify major pedicles after finishing parenchymal division with Ligasure or harmonic scalpel. The major pedicles are transected using vascular endo-stapler under direct vision through the small incision, and the specimen is delivered at once without breaking.

Advantages over full laparoscopic subtotal gastrectomy

First, the greater and lesser omentum are dissected using this technique much easier and quicker. Second, the stomach is transected and gastrojejunostomy is performed by using open staplers, which are much easier, quicker, more reliable, and less expensive than using endo-staplers in full laparoscopic subtotal gastrectomy. Third, a big and hard duodenal ulcer like the one in our cases is not the indication for full laparoscopic technique, because such duodenal ulcer can neither be transected, nor can the stump be closed with staplers.

The design of the approach for HALS in resection of abdominal large viscera is based on the minimal abdominal wound “1 port + 2 trocars”, as well as the minimal surgical intervention, combining the laparoscopic benefits with the advantages of a conventional open technique. Even though the target organs include parenchymatous and hollow viscera, the rules of resection are the same, which allow for first dissecting the peripheral structures, and finally transecting the core vessel pedicles. This technique is minimal in access, safe, and rapid in laparoscopic resection of abdominal large viscera.

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Treatment of hepatoma with liposome-encapsulated adriamycin administered into hepatic artery of rats

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Abstract

AIM: To observe the therapeutic effects of liposome-encapsulated adriamycin (LADM) on hepatoma in comparison with adriamycin solution (FADM) and adriamycin plus blank liposome (ADM + BL) administered into the hepatic artery of rats.

METHODS: LADM was prepared by pH gradient-driven method. Normal saline, FADM (2 mg/kg), ADM+BL (2 mg/kg), and LADM (2 mg/kg) were injected *via* the hepatic artery in rats bearing liver W256 carcinosarcoma, which were divided into four groups randomly. The therapeutic effects were evaluated in terms of survival time, tumor enlargement ratio, and tumor necrosis degree. The difference was determined with ANOVA and Dunnett test and log rank test.

RESULTS: Compared to FADM or ADM + BL, LADM produced a more significant tumor inhibition (tumor volume ratio: 1.243 ± 0.523 vs 1.883 ± 0.708 , 1.847 ± 0.661 , $P < 0.01$), and more extensive tumor necrosis. The increased life span was prolonged significantly in rats receiving LADM compared with FADM or ADM+BL (231.48 vs 74.66, 94.70) ($P < 0.05$).

CONCLUSION: The anticancer efficacies of adriamycin on hepatoma can be strongly improved by liposomal encapsulation through hepatic arterial administration.

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Key words: Adriamycin; Liposome; Hepatoma

INTRODUCTION

Adriamycin (ADM) is extensively used to treat patients with hepatocellular carcinoma (HCC). *In vitro* studies have verified that the cytotoxic effect of ADM depends on the concentration and duration of exposure^[1]. Unfortunately, intravenous administration (iv) of a high-dose ADM is often associated with acute toxicity, such as myelosuppression, immunosuppression, and dose-cumulative cardiotoxicity. As a result, drug concentrations in hepatomas are limited. During the past few years, many researchers have reported on drug delivery systems for cancer chemotherapy that aim at the specific targeting of antitumor agents to tumor cells or tumor tissues, thus enhancing the efficacy of chemotherapy as well as reducing its toxicity^[2]. Among them, liposomes have drawn much attention for their excellent bioavailability, biodegradability, and targeting, characteristically, to the reticuloendothelial system (RES), especially the liver and spleen^[3].

On the other hand, liver primary tumors receive approximately 90% of their blood supply from the hepatic artery. Therefore, transhepatic arterial chemotherapy (TAC) is now widely recognized as an effective means for the treatment of HCC. With TAC, a much higher drug concentration in the tumor-residing zone can be achieved. It was reported that administration of ADM via the hepatic artery (ia) was able to increase the tumor ADM concentration three-fold compared to intravenous (iv) administration^[4]. In patients with cancers, the ia administration of ADM reduced the plasma AUC by about 30%^[5]. On the basis of these evidences, we hypothesized that a further significant therapeutic effect could be expected by combining TAC and liposome techniques. The current study was carried out to observe the therapeutic effects of liposome-encapsulated adriamycin (LADM) on hepatoma in comparison with the treatment results of adriamycin solution (FADM) and adriamycin plus blank liposome (ADM + BL) administered into the hepatic artery in rats.

MATERIALS AND METHODS

Liposomal adriamycin (LADM) preparation and characterization

Large unilamellar vesicles (LUVs) were prepared by the extrusion method described by Hope *et al.*^[6]. Appropriate amounts of lipid mixtures (EPC/Chol, 55:45 mol/mol) in chloroform were dried under a stream of nitrogen gas to form a homogeneous lipid film. The trace amount of solvent was then removed under vacuum overnight. The lipid film was hydrated in a low pH citrate buffer (pH 4.0, 300 mmol/L) by vortex mixing. The resulting multilamellar vesicles (MLVs) were frozen/thawed (liquid nitrogen/55°C) 5 times and extruded 10 times at 55°C through two stacked 100 nm polycarbonate filters (Nuclepore) employing an extrusion device (Lipex Biomembranes, Inc., Vancouver, BC, Canada). ADM was then encapsulated by pH gradient-driven method as described previously^[7].

The final product looked like a reddish, semitransparent, colloidal solution. Under electron microscope, LADM showed a global, regular contour with homogeneous size and distribution. The average 120 nm diameters of the liposomes were determined by quasi-elastic light scattering (QELS) using a Nicomp 370 submicrom particle sizer (Sante Barbara, CA, USA) as shown in Figure 1. EPC and Chol were quantified by high performance liquid chromatography (HPLC) method (Table 1). HPLC analysis was performed using a Perkin Elmer HPLC with a PE 200 LC pump, a PE ISS 200 Advanced LC sample Processor, PE 900 Series Interface and an Evaporative Light Scattering Detector (ELSD, Shimadzu). A YMC, PVA 5 m normal phase column was used in this study. The chromatograph was run in a gradient mode at a flow rate of 1 mL/min with the following program, where solution A contained chloroform-isopropanol (50:50, v/v) and solution B contained chloroform-isopropanol-water (36:55:9, v/v).

The drug-to-lipid ratio was 0.25. The drug-embedding ratio was more than 98%. Encapsulation efficiency was calculated as the percentage of ADM incorporated into liposomes relative to the initial amount of ADM in solution as shown in the equation below. The free ADM was separated by elution over a Sephadex G-50 column. Then the free ADM and liposomal ADM were determined by HPLC.

$$\text{Encapsulation efficiency (\%)} = \frac{(\text{adriamycin/lipid})_{\text{Encapsulated}}}{(\text{adriamycin/lipid})_{\text{total}}} \times 100$$

The liposomal particle sizes were identical before and after drug loading, and ADM encapsulated liposomes were very stable at room temperature for three days. There was no significant leakage of ADM from liposomes. Just before use, normal saline was added to adjust the final concentration of total ADM to 1.0 mg/mL. Free ADM (FADM) was prepared by dissolution of ADM in normal saline at the same concentration.

Animals and anesthesia

Sixty male Wistar rats weighing 230-270 g (mean, 250 g) were provided by Laboratory Animal Center of Fourth

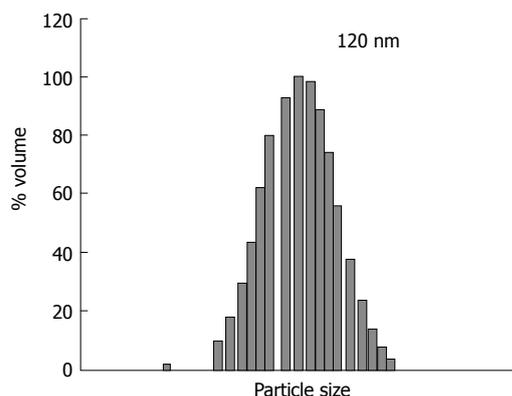


Figure 1 The particle size of prepared liposome with EPC/Chol at 55:45 molar ratio by the extrusion method. The average diameters were determined by QELS.

Table 1 Detailed solvent gradient program for analysis of EPC and Chol by HPLC

Time (min)	0-3	3-11	11-11.5	11.5-30
A%	100	0	0	100
B%	0	100	100	0

Solution A: Chloroform-isopropanol (50:50, v/v); Solution B: Chloroform-isopropanol-water (36:55:9, v/v). EPC: egg phosphatidyl choline; Chol: Cholesterol.

Military Medical University and randomly divided into 4 groups, 15 in each. Sumianxin (Changchun Agricultural Pastoral University) was used as anesthetic.

Establishment of hepatoma model

One milliliter of suspension containing 10^7 Walker-256 (W256) carcinosarcoma cells (Shanghai Medical Industrial Institution) was injected into the thigh muscle of a carrier rat (not included in experimental rats). One week after inoculation, a palpable tumor was found at the injection site. Viable tumor tissue was excised under sterile conditions and soaked in 20 mL of Hanks balanced salt solution. Tissue was cut into approximately 1 mm × 1 mm × 1 mm fragments. Experimental rats were anesthetized with intramuscular injection of Sumianxin at 0.2 mL/kg. Median incision beneath the metasternum was made and the liver was exposed. The tumor fragment was implanted into the left liver lobe.

Administration through hepatic artery

On the 7th d after tumor implantation, all animals received laparotomy again. The longest (a) and shortest (b) diameters of the tumor were measured. The tumor volume was calculated as

$$\frac{a \times b^2}{2}$$

By cannulation method described previously^[8], normal saline (NS), FADM, LADM, or ADM mixed with blank liposomes (ADM+BL) were injected into the hepatic artery of rats in groups 1-4 respectively. The dose of ADM in each formulation was 2.0 mg/kg body weight. The concentration of ADM was 1.0 mg/mL.

Assessment of therapeutic effect

Tumor growth inhibition: Seven days later, all rats received the third laparotomy. The longest (a) and shortest (b) diameters of the tumor were measured again and the tumor volume after drug administration was calculated. The tumor volume ratio (TVR) was calculated as

$$\text{TVR} = \frac{\text{Tumor volume after administration}}{\text{Tumor volume before administration}}$$

Tumor necrosis degree: Seven random rats in each group were killed and anatomized. Hepatoma was removed completely and fixed in 10% formalin. Three 5 μm thick sections from each tumor were sliced on the maximal transverse plane and mounted on glass slides overnight at room temperature. After HE staining, the sections were examined under microscope. According to the percentage of necrosis area, tumors were graded according to the following criteria: I, 0%-30%; II, 30%-70%; III, 70%-100%.

Increased life span: Survival time of the remaining 8 rats in each group was recorded. The mean survival time of NS group was reckoned as control. Increased life span (% ILS) was calculated as

$$\% \text{ILS} = \left(\frac{\text{Mean survival of treated group}}{\text{Mean survival of control group}} - 1 \right) \times 100\%$$

Statistical analysis

SPSS 10.0 for windows was used for statistical analysis. Statistical significance was tested by ANOVA and Dunnett test for tumor growth inhibition, log rank test for survival time. $P < 0.05$ was considered statistically significant.

RESULTS

Tumor growth inhibition

No difference in tumor volume was found among the four groups before treatment ($P > 0.05$, Table 2). After treatment, the tumor grew rapidly in rats which received NS. The mean tumor volume was 31.55 times greater than that before treatment. Metastases were observed in about half of these rats. In contrast, the rate of tumor growth lowered apparently in rats which received FADM or ADM + BL ($P < 0.01$). No difference between FADM and ADM + BL was observed ($P > 0.05$). The slowest tumor growth was found in rats which received administration of LADM. The mean tumor volume ratio on the 7th day after LADM infusion was 1.243. Statistics indicated that LADM produced a further significant tumor inhibition, as compared with FADM or ADM + BL ($P < 0.01$). In addition, no metastasis was found in rats receiving LADM.

Tumor necrosis degree

Under microscope, W256 tumor cells of rats that received NS showed extensive proliferation and active mitoses. Small areas of necroses were observed in the center of tumor tissue accompanied with a few inflammatory cells (Table 3). Moderate to severe necroses were found in tumors of rats that received FADM or ADM+BL. Grade III of tumor necroses was found in 4 of 7 tumors after

Table 2 Tumor volume (V) and tumor volume ratio (TVR) after treatment (mean \pm SD)

Group	V (cm ³)		TVR
	Before treatment	After treatment	
NS	0.086 \pm 0.049	2.521 \pm 0.840	31.550 \pm 7.975
FADM	0.083 \pm 0.035	0.149 \pm 0.072	1.883 \pm 0.708 ^b
ADM + BL	0.079 \pm 0.036	0.136 \pm 0.069	1.847 \pm 0.661 ^b
LADM	0.084 \pm 0.049	0.105 \pm 0.062	1.243 \pm 0.523 ^d

^b $P < 0.01$ vs NS; ^d $P < 0.01$ vs FADM, ADM + BL.

Table 3 Tumor necrosis degree after treatment

Grade	NS	FADM	ADM + BL	LADM
I	6	2	1	0
II	1	3	4	3
III	0	2	2	4 ¹

¹ Complete necrosis in 2 cases.

Table 4 Mean survival time and increased life span after treatment (mean \pm SD)

Group	Mean survival time (d)	% ILS
NS	11.88 \pm 2.80	-
FADM	20.75 \pm 6.34	74.66 ^b
ADM + BL	23.13 \pm 7.75	94.70 ^b
LADM	39.38 \pm 11.15	231.48 ^a

% ILS: Increased life span. ^a $P < 0.05$ vs FADM, ADM + BL; ^b $P < 0.01$ vs NS.

administration of LADM, including 2 cases of complete tumor necrosis.

Increased life span

Compared with the survival time (11.88 d) of control rats, survival time was greatly prolonged in rats that received LADM (mean, 39.38 d), or FADM (mean, 20.75 d), or ADM+BL (mean, 23.13 d) (Table 4). LADM prolonged the life span by 231.48%, which was significantly longer than FADM (74.66%) and ADM + BL (94.70%) ($P < 0.01$).

DISCUSSION

Hepatocellular carcinoma (HCC) is by far the third most common cause of cancer mortality throughout the world, especially in the Asian-Pacific region, and its incidence continues to increase. On the other hand, HCC has a high expression rate of multi-drug resistance gene (MDR1) and consequently high levels of P-glycoprotein (P-gp), resulting in its insensitiveness to most chemotherapeutic drugs^[9]. Few agents show response rates (RRs) above 20%. ADM is one of the most successful anticancer agents to date for the treatment of HCC. Early phase II trials and case series

reported RRs ranging from 25% to 100% with single-agent ADM^[10]. However, despite its excellent antitumor activity, ADM has a relatively low therapeutic index, and its clinical utility is frequently limited due to acute and chronic toxicities such as myelosuppression, immunosuppression, and dose-cumulative cardiotoxicity. A retrospective analysis of three phase III trials of patients treated with ADM in combination with other cytotoxic agents or radiation therapy demonstrates that ADM-associated cardiac events may occur more frequently and at lower cumulative doses than previously reported^[11]. Encapsulation of ADM into liposomes may minimize the side effects and enhance the antitumor efficacy by altering its pharmacokinetics and biodistribution pattern. Growing evidence supports the use of LADM as a substitute for ADM to increase the therapeutic index by maintaining the antitumor efficacy while improving the safety profile^[12,13]. After intravenous (iv) administration, LADM is predominantly uptaken by the reticuloendothelial system (RES), especially by the liver and spleen^[14].

The results of our experiments demonstrated that the antitumor activity of ADM could be markedly enhanced when the drug was encapsulated in liposomes and administered into the hepatic artery. Equivalent or similar effects could not be obtained using FADM or ADM + BL. Compared with FADM or ADM + BL, LADM produced a more significant tumor inhibition and more extensive tumor necrosis. The average tumor volume on the 7th day after treatment with LADM was 0.105 cm³, about 70% of 0.149 cm³ of the volume after treatment with FADM. The survival time of rats that received LADM was evidently increased. Compared with saline controls, LADM prolonged the life span by 231.48%, which was significantly longer than FADM (74.66%) and ADM + BL (94.70%).

The likely explanations for the improved therapeutic activity of LADM are as follows. First, W256 carcinosarcoma proliferated rapidly with abundant vasculogenesis, which strongly increased the uptake of LADM by tumor tissues after ia administration. In addition, the deformity of tumor vessels caused significant retention of LADM in tumor tissues. Secondly, it has been testified that the cytotoxic effect of ADM depends on the concentration and duration of exposure^[1]. Increasing ADM concentration in tumor cells or slowing its elimination could certainly enhance its antitumor efficacy, both of which could be met after ia administration of LADM. Thirdly, encapsulating the drug into liposomes might modify its distribution pattern in tissues. After being administered into rats, liposomes were taken up selectively by RES, such as the liver, spleen, and bone marrow^[14]. Accumulation of biodegradable liposomes with associated drugs in Kupffer cells would create a gradient of drug concentration for a massive and prolonged diffusion of the free drug towards neoplastic tissues. Fourthly, the tiny size of the liposome could increase its contact areas significantly, which might result in an apparent improvement in its bioavailability.

We did not include iv administration in the present

experiment because the ia route is much more efficient than iv in the treatment of liver malignancies. Different from the liver parenchyma, which receives more than 70% of its blood supply from the portal vein and the rest from the hepatic artery, hepatoma receives approximately 90% of its blood supply from the hepatic artery. The uniqueness of liver blood supply determines the major difference between the two routes of administration. The difference does not warrant repeat comparison in our study.

Our experiments support the hypothesis that the therapeutic effect of TAC could be dramatically enhanced by combination with nanotechnology. We conclude that liposomes can be used as a promising drug carrier in TAC for the treatment of liver primary and metastatic tumors.

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Role of detection of microsatellite instability in Chinese with hereditary nonpolyposis colorectal cancer or ordinary hereditary colorectal cancer

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Abstract

AIM: To detect microsatellite instability (MSI) in patients with hereditary nonpolyposis colorectal cancer or ordinary hereditary colorectal cancer and to provide criteria for screening the kindreds with hereditary nonpolyposis colorectal cancer at molecular level.

METHODS: MSI was detected in the specimens from 20 cases with HNPCC, 20 cases with ordinary hereditary colorectal cancer and 20 cases with sporadic colorectal cancer by means of polymerase chain reaction-single strand conformation polymorphism.

RESULTS: The positive rate of MSI was 85% (17/20) in HNPCC group, 40% (8/20) in ordinary hereditary colorectal cancer group and 10% (2/20) in the sporadic colorectal cancer group respectively. The differences were significant. The mean ages of the three groups were 43.6, 52.2, and 61.8 years respectively, which increased gradually. The incidence of right hemicolon cancer was 64.7%, 37.5%, and 0% respectively, which decreased gradually and had significant difference. The expression ratio of BAT26 and BAT25 was 94.1% respectively, which was highest in the 5 gene sites studied. The incidence of poorly differentiated adenocarcinoma was 70.6% in HNPCC group among high frequency microsatellite instability (MSI-H), which was higher than the other two groups, which had 50% and 50% respectively.

CONCLUSION: The incidence of MSI-H is higher in HNPCC group. The detection of MSI is simple and economical and has high correlation with the clinicopathologic feature of HNPCC and can be used as a screening method to detect the germ line mutation of the mismatch repair gene.

INTRODUCTION

Hereditary nonpolyposis colorectal cancer (HNPCC) is an autosomal dominant inheritance syndrome, with a penetrance as high as 70%-80%^[1], and accounts for about 5%-15% of all colorectal cancer^[2]. The molecular genetic basis of the disease is germ line mutation of the mismatch repair (MMR) gene, which causes failure of the DNA MMR system to repair errors that occur during the replication of DNA and results in alterations in the length of simple, repetitive microsatellite sequences and so called microsatellite instability (MSI). MSI may reflect the mutation of the MMR gene indirectly and can be used as a means of screening gene mutation of the MMR gene^[3,4]. Recently, studies showed most patients with HNPCC have MSI^[5,6] and the ratio is higher than that of patients with sporadic colorectal cancer^[7,8]. In the current study, we tested microsatellites of the former paraffin-embedded tissue by the method of polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) of the Chinese patients who fulfilled the criteria for HNPCC and ordinary hereditary colorectal cancer and tested its application value in the clinic.

MATERIALS AND METHODS

Patients

HNPCC group (group A): 20 patients (12 men, 8 women, mean age 48 years, range 32-70 years) who fulfilled the criteria for HNPCC of Chinese people^[9] were selected and their family histories were obtained by follow-

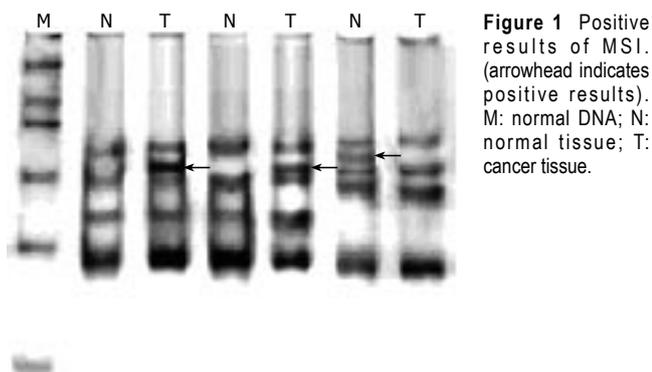


Figure 1 Positive results of MSI. (arrowhead indicates positive results). M: normal DNA; N: normal tissue; T: cancer tissue.

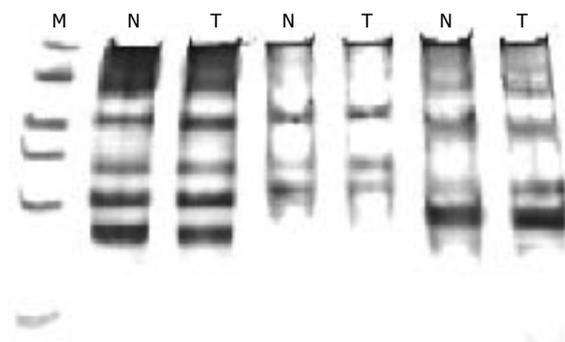


Figure 2 Negative results of MSI (results of 3 pairs are all negative). M: normal DNA; N: normal tissue; T: cancer tissue.

up study. Among them 9 cases were with carcinoma of ascending colon, 2 cases with carcinoma of transverse colon, 1 case with carcinoma of descending colon, 2 cases with carcinoma of sigmoid colon and 6 cases with carcinoma of rectum.

Ordinary hereditary colorectal cancer group (group B): 20 patients (13 men, 7 women, mean age 61 years, range 30-83 years) who fulfilled the criteria for ordinary hereditary colorectal cancer of Chinese people^[10] were selected. Among them 5 cases were with carcinoma of ascending colon, 3 cases with carcinoma of transverse colon, 1 case with carcinoma of descending colon, 2 cases with carcinoma of sigmoid colon and 9 cases with carcinoma of rectum.

Sporadic colorectal cancer group (group C): 20 patients (10 men, 10 women, mean age 65 years, range 42-80 years) who were diagnosed with colorectal cancer by pathology and with no family history were selected. Among them 5 cases were with carcinoma of ascending colon, 4 cases with carcinoma of sigmoid colon and 11 cases with carcinoma of rectum.

Methods

For MSI analysis, normal and tumor tissues of the three groups were embedded with paraffin, 4-5 slides of tissue with thickness of 4 μm were sliced and stained with HE. Normal and tumor tissues were selected with microscopy. They were transferred to the EP tubes which contained 150 μL cell lysates. Then DNAs of the normal and tumor tissues were extracted with DNA extraction kit. The primers of the 5 microsatellite loci of HNPCC (BAT26, BAT25, D2S123, D5S346 and D17S250) were selected according to recommendation of the international cooperation organization (Table 1). The primers were synthesized by TAKARA Company.

The reaction system: It was consisted of a total volume of 25 μL with 3 μL template DNA, 0.5 μL forward and reverse primers, 10 \times Buffer 2.5 μL , dNTP 2.0 μL , TaqE 0.2 μL , DMSO 1.0 μL , ddH₂O 15.3 μL . The PCR program started with a 95°C denaturation for 5 min, then at 94°C for 40 s, 53°C for 30 s followed by 35 cycles of annealing at 72°C for 1 min, and extension at 72°C for 40 s, finally an extension at 72°C for 10 min. The PCR products were analyzed by electrophoresis on 1.5% agarose gels containing ethidium bromide.

Detection of MSI: Single strand conformation polymorphism (SSCP) was used to analyze MSI. The PCR products

of normal and tumor tissues were mixed with the same volume of alkaline buffer, then started with 97°C denaturation for 10 min and put in the mixture of water and ice for 5 min. Then electrophoresis was performed vertically on the 10% nondenaturing polyacrylamide gel (constant power; 60 W) for about 4 h. When the indicating straps reached the inferior margin of the gel or the tracer agent disappeared, silver staining, coloration, fixation and termination were performed. When the film dried 24 h later, the imaging was observed.

Results assessment: MSI was defined by the presence of novel bands following PCR amplification of tumor DNA, which were not present in PCR products of the corresponding normal DNA. If more than 2 of them showing positive results, it was defined as high frequency microsatellite instability (MSI-H), and if only 1 of them showing positive result, it was defined as low frequency microsatellite instability (MSI-L), and if none of them showing positive result, it was named microsatellite stable (MSS).

RESULTS

The results of detection of MSI are shown in Figures 1 and 2. and Tables 1 and 2.

The relationship among MSI-H, mean age of the patients and the sites of cancer are shown in Table 3.

The ratio of every locus occupying in the MSI-H is shown in Table 4.

The clinical pathological characteristics of colorectal cancer are as follows: in the 17 cases with MSI-H of group A, 7 cases had multiple cancers, among which 4 cases were with multiple cancers simultaneously, 3 cases were with multiple cancers at different times. As to groups B and C, none of the patients were with multiple cancers. There were 70.6% (12/17) of poorly differentiated adenocarcinoma in group A, which was more than that of groups B (50%, 4/8) and C (50%, 1/2).

DISCUSSION

Microsatellites are long stretches of apparently redundant DNA between genes by 2-6 nucleotides tandemly arrayed, within which repetitive sequences may be found. Repetition of two bases(CA/GT) is most common. Under normal conditions, the repetitive sequence is constant but

Table 1 Primer sequence of MSI and annealing temperature

Locus	Primer sequence	Annealing temperature (°C)
BAT26	TGACTACTTTTGACTTCAGCC	53.9
	AACCATCAACATTTTAAACCC	
BAT25	TCGCCTCAAGAATGTAAGT	53.4
	TCTGCATTTTAACTATGGCTC	
D2S123	AAACAGGATGCCTGCCTTTA	57.4
	GGACTTTCACCTATGGGAC	
D5S346	ACTCACTCTAGTGATAAATCGGG	54.0
	AGCAGATAAGACAGTATTACTAGTT	
D17S250	GGAAGAATCAAATAGACAAT	53.6
	GCTGGCCATATATATTTAAACC	

Table 2 Detection of MSI in three groups (%)

Group	n	MSI-H	MSI-L	MSS
A	20	85 (17/20)	10 (2/20)	5 (1/20)
B	20	40 (8/20)	35 (7/20)	25 (5/20)
C	20	10 (2/20)	30 (6/20)	60 (12/20)

MSI-H: high frequency microsatellite instability; MSI-L: low frequency microsatellite instability; MSS: microsatellite stable.

highly polymorphic and is an ideal mark for localization of genes. Cells lacking normal DNA mismatch repair acquire mutations that change the length of nucleotide repeat sequences, termed microsatellite instability (MSI). MSI is the pattern of manifestation of MMR and has a close relationship with HNPCC^[11,12], therefore the mutation of MMR results in the incidence of MSI. About 70%-90% of the patients with HNPCC had MSI and only 10%-15% occurred in patients with the sporadic colorectal cancer. Although the Amsterdam Criteria (the International Collaborative Group established in 1991) is now used as the diagnostic criteria for HNPCC clinically, its determination depends mainly on family history and clinical symptoms and cannot reflect the true morbidity of the disease and predict the occurrence of HNPCC. Therefore the detection of mutation of MMR genes has become the golden standard for diagnosis of HNPCC. However, the MMR gene is very large, it has no mutational hot spot, and the cost of detection is expensive, thus it cannot be used as a routine means for screening of HNPCC family at present. As a phenotype of MMR gene mutation, MSI can be used as criterium for detection of HNPCC, especially in patients whose family history is not complete and who do not fit the criteria of HNPCC clinically. Therefore, DNA is extracted from the tumor tissue for detection of MSI. When a patient of MSI-H is diagnosed, detection of MMR gene should be done. As to the choice of loci of the microsatellite instability, 5 loci of microsatellite are used for PCR amplification according to the recommendation of the International Collaborative Group and the International Cancer Research Institute and the Collaborative Group of the Chinese Genetic Colorectal Cancer. However, number of the microsatellites detected is not constant, from less than 3 loci to more

Table 3 Relationship among MSI-H, mean age of the patients and the sites of cancer (%)

Group	n	MSI-H	Mean age (yr)	Right hemicolon	Left hemicolon
A	20	17 (85)	43.6	11 (64.7)	6 (35.3)
B	20	8 (40)	52.2	5 (62.5)	3 (37.5)
C	20	2 (10)	61.8	0 (0)	2 (100)

Table 4 Ratio of every locus occupying in the MSI-H (%)

Locus	Positive ratio of MSI-H	Positive ratio of MSI
BAT26	94.1 (16/17)	85 (17/20)
BAT25	94.1 (16/17)	90 (18/20)
D2S123	64.7 (11/17)	55 (11/20)
D5S346	52.9 (9/17)	50 (10/20)
D17S250	35.3 (6/17)	40 (8/20)

than 100 loci. The criteria for the number and ratio of the positive locus of MSI tumor are not uniform. Therefore the results of many different researchers could not be compared with each other.

In this study, we amplified 5 loci of microsatellite by PCR according to the criteria of the International Collaborative Group and the International Cancer Research Institute and found the positive ratio was as high as 85% for MSI-H patients with HNPCC, which is in accordance with other research. We found the 5 loci were ideal for detection of MSI of HNPCC patients. It reflected the mutation of MMR gene objectively and could be used as a reliable indicator for screening of HNPCC family just before gene sequencing. If other loci were selected, the type of MSI could be decided by the percentage of positive locus, and MSI-H could be diagnosed when the percentage of positive loci was more than 30%-40% while MSI-L was diagnosed when the percentage of positive loci was less than 30%. As to which loci should be selected, there are a variety of viewpoints. Some scholars think 1-2 microsatellite loci are enough. Hoang^[13] believes BAT26 has high correlation with MSI, and their concordance rate is very high. Because the detecting process is relatively simple, Stone^[14] detected MSI from tumor specimen using BAT26 directly and recommended that it should be used more extensively. Our study found the positive rate of BAT26 was as high as 94.1%, indicating its high sensitivity for MSI-H detection. At the same time, the positive rate of BAT25 was also 94.1%. Their annealing temperature was close to each other and could be carried out in the same reaction system. The combined application of BAT26 and BAT25 may elevate the specificity of the experiment.

Moreover, our research found that about 40% of the patients with ordinary hereditary colorectal cancer were classified as MSI-H, although it was lower than that of the HNPCC group, it was higher than that of the sporadic colorectal cancer group (the positive rate was 10%), suggesting that about 30% of the patients with ordinary hereditary colorectal cancer had the characteristics of HNPCC. Whether these patients were potential HNPCC

patients needs further study. MSI has been shown to have a close relationship with the onset age of the patients in many recent studies. MSI-H shows good consistency with early onset of age. Edmonston^[15] found that the patients whose onset of age was less than 41 years were all MSI-H positive, and the youngest onset age of MSI-H was less than 26 years. Terdiman^[16] found that in the different HNPCC families, the final diagnosis age of MSI-H was young and there were more family members with HNPCC than other families, and the ratio of multiple cancers or other HNPCC-related cancer increased. Our research revealed that the mean onset age of MSI-H in the HNPCC group was 43.6 years, which was younger than that of the ordinary hereditary colorectal cancer group (52.2 years) and the sporadic colorectal cancer group (61.8 years). The incidence of multiple cancers or other HNPCC-related cancers of HNPCC group was higher than other groups. In addition, MSI had a close relationship with the sites of carcinoma. The carcinoma of MSI-H, either of HNPCC or of sporadic colorectal cancer, was all likely to occur at the proximal half of a colon. Just as Altonen's study showed^[17], 62% of the tumors occurred at the proximal hemicolon of the patients with HNPCC while 38% occurred at the distal hemicolon, suggesting that MSI had a close relationship with cancer of right hemicolon and may play a fundamental role in the pathology of cancer of right hemicolon. Our research found the incidences of right hemicolon cancer of the HNPCC group and the ordinary hereditary colorectal cancer group were 64.7% and 62.5% respectively, which were higher than those of left hemicolon cancer. There was no cancer of right hemicolon found in the sporadic colorectal cancer group, because the occurrence of rectum cancer was higher in North China and the bias was related with the cases selected. Our research suggests that MSI had high correlation with the clinical and pathological characteristics of the HNPCC.

At present, there have been few reports about MSI in typical cases of Chinese people and no clinical data about MSI in patients with ordinary hereditary colorectal cancer. Our research demonstrates that the incidence rate of MSI-H in patients with HNPCC, the mean onset age, the site of carcinoma occurrence, and the clinical and pathological characteristics of patients with HNPCC are all different from that of patients with sporadic colorectal cancer. Ordinary hereditary colorectal cancer is a special type between HNPCC and sporadic colorectal cancer. It has partial characteristics of HNPCC but is not the same as HNPCC and has many differences from sporadic colorectal cancer. Whether it has a special molecular genetic mechanism needs further study. In addition, the survival rate of patients with HNPCC is higher than that of patients with sporadic colorectal cancer, which is due to the early diagnosis of some patients with HNPCC^[18,19]. Recently, studies reported that MSI induced by abnormal expression of MMR may affect the therapeutic effect of chemotherapy and the prognosis of patients with colorectal cancer^[20-22]. Therefore, as an important screening method for HNPCC, MSI has a promising prospect in clinical application.

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RAPID COMMUNICATION

Pharmacodynamic and kinetic effect of rabeprazole on serum gastrin level in relation to CYP2C19 polymorphism in Chinese Hans

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Abstract

AIM: To observe the pharmacokinetics and pharmacodynamics of rabeprazole and compare serum gastrin concentrations in different CYP2C19 genotype groups.

METHODS: The CYP2C19 genotype status of Chinese Han healthy volunteers was determined by polymerase chain reaction-restriction fragment length polymorphism method. Twenty *H pylori*-negative healthy subjects voluntarily participated in the study. They were divided into the following three groups: homozygous extensive metabolizers (homEM), heterozygous extensive metabolizers (hetEM) and poor metabolizers (PM). After they orally received rabeprazole 20 mg once daily in the morning of d 1 and d 8, blood samples were collected at various time-points until 24 h after administration and intragastric pH values were monitored for 24 h by Digitrapper pH. Serum gastrin concentrations were measured by radioimmunoassay. Serum concentrations of rabeprazole were measured by high performance liquid chromatography.

RESULTS: The mean AUC values for rabeprazole after a single and repeated doses were significantly different between the homEM and PM groups, but not between the homEM and hetEM, or the hetEM and PM groups. No significant differences in intragastric pH medians were observed among the three different genotype groups after a single dose or repeated doses. The ratio of pH medians between d 1 and d 8 ranged from 84% to 108%. The mean gastrin AUC values were also different among the three genotype groups, with a relative ratio of 1.0, 1.2 and 1.5 after a single dose and 1.0, 1.5 and 1.6 after repeated doses in the homEM, hetEM and PM

groups, respectively. The gastrin AUC values among the three different genotype groups showed no significant difference either after a single dose or repeated doses. The subject who had lower intragastric acidity showed higher serum gastrin levels and concentrations of rabeprazole.

CONCLUSION: In Chinese Han healthy people, the pharmacokinetics of rabeprazole are dependent on the CYP2C19 genotype status, but acid-inhibitory efficacy of rabeprazole and the gastrin level are not influenced significantly.

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Key words: CYP450; Pharmacokinetics; Pharmacodynamics; Proton pump inhibitors

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INTRODUCTION

CYP450 2C19 is a genetically determined enzyme. Its phenotypes are either poor metabolizer (PM) or extensive metabolizer (EM)^[1,2] in the general population. When CYP2C19 is the main metabolism enzyme of a drug, the pharmacokinetics and pharmacodynamics of the drug are different between PMs and EMs. Rabeprazole (RPZ), a kind of the newer proton pump inhibitors (PPIs), has been reported to be metabolized mainly *via* a non-enzymatic pathway, with only minor CYP2C19 and CYP3A4 involvement. The pharmacokinetics of RPZ are expected to be less influenced by the CYP2C19 phenotype than those of omeprazole^[3-6]. However, it is not clear whether the pharmacokinetics and pharmacodynamics of RPZ depend on the CYP2C19 genotype status in Chinese Han people. On the other hand, it is well known that gastrin is secreted from G cells in the antrum of the stomach^[7], and an inhibition of gastric acid secretion by PPIs can stimulate gastrin release from G cells^[8]. Until now, little is known about the kinetics and pharmacodynamic effect

of RPZ on gastrin levels with respect to the polymorphic CYP2C19 in Chinese Han people. Therefore, in the present study, we observed the metabolic characteristics and pharmacodynamics of RPZ and serum gastrin levels after the single and 8-d repeated doses in the different CYP2C19 genotype groups. It showed that acid-inhibitory efficacy of rabeprazole and the gastrin levels were not influenced significantly by CYP2C19 genotype.

MATERIALS AND METHODS

Subjects and CYP2C19 genotypes

Twenty healthy male subjects who were negative for *H pylori* infection were enrolled in the study. None of the subjects consumed alcohol or smoked, and none had taken any drugs at least 4 wk before or during the study. The protocol was approved in advance by the ethic committee of Anhui Medical University. Written informed consent was obtained from each of the subjects before participation in the study.

Genomic DNA was extracted from leucocytes of each individual using a commercially available kit (Promega, USA). The genotyping of CYP2C19, including CYP2C19*wt, CYP2C19*m1 and CYP2C19*m2, was performed by polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP)^[9]. The age of the subjects ranged from 20 to 23 years and body weight from 57 to 63 kg. Subjects were genotypically classified into the following three groups: homozygous extensive metabolizer group (homEM, $n = 7$), heterozygous extensive metabolizer group (hetEM, Wt/m1, $n = 4$; Wt/m2, $n = 2$) and poor metabolizer group (PM, m1/m1, $n = 7$).

Study protocol

Subjects were initially screened for *H pylori* infection by a serological test (Dot-immunogold kit, Lanbo Bio-Tech Institute, China) and ¹³C-urea breath test. Each healthy volunteer received 20 mg RPZ (Pariet, Eisai Co. Ltd, Tokyo, Japan) orally for 8 d. The medications were taken once daily at 8:00 am. The 24-h intragastric pH monitoring and the measurement of plasma concentration of RPZ and gastrin level were performed on d 1 and d 8. Blood samples were collected before and 0.5, 1, 1.5, 2, 3, 5, 7, 10, 12, 24 h after administration on d 1 and d 8. After collection, the blood samples were immediately centrifuged at 4000 r/min for 10 min. One hundred microliter 1% diethylamine solution was added to the 1 mL plasma sample for determination of the concentration of RPZ. All samples were stored at -80°C until assayed. Plasma levels of RPZ were measured by high performance liquid chromatography^[10,11]. Serum gastrin levels were measured using radioimmunoassay (Gastrin-RIA kit, North Bio-Tech Institute, China).

Intragastric pH measurement

After overnight fasting, a glass electrode was inserted transnasally and placed about 5 cm below the cardia. The electrode was calibrated with standard buffers (pH 1.07 and 7.01) before recording the pH with a DigiTrapper pH (Medtronic). Intragastric pH recordings started after the oral dose of RPZ at 8:00 am on d 1 and d 8. Two standard

Table 1 AUC of RPZ 20 mg after a single and repeated doses

	AUC ^a (μg/L per h) d 1	AUC ^a (μg/L per h) d 8
homEM	1150.24 ± 327.34	1445.28 ± 205.16
hetEM	1539.42 ± 190.29	1640.91 ± 249.51
PM	2015.38 ± 588.88	2495.61 ± 738.61

^a $P < 0.05$ homEM vs PM.

meals (noon, 18:00 pm), prepared at the hospital, were provided for each subject.

Pharmacokinetic analysis

Pharmacokinetic analysis was performed in a model-independent manner, and non-compartmental kinetic parameters (AUC) were calculated with 3P87 software. The area under concentration-time curve (AUC) for RPZ and gastrin in serum was shown from zero to 24 h (AUC₀₋₂₄).

Statistical analysis

The data are given as mean ± SD in all analyses. Differences in AUC and intragastric pH values between three genotype groups were compared using one-way analysis of variance (ANOVA) combined with the least significant method (LSD). To determine whether the gastrin concentrations and AUC values were increased from a single dose to repeated doses, the paired *t*-test was used. Statistical calculations were performed by SPSS 10.0 software. *P* value of less than 0.05 was considered to be statistically significant.

RESULTS

CYP2C19 genotype and AUC of RPZ

The mean AUC of RPZ is shown in Table 1. The mean AUC values for RPZ after a single dose differed among the three genotype groups, with a relative ratio of 1.0, 1.3 and 1.8 in the homEM, hetEM and PM groups, respectively. The mean AUC values for RPZ after repeated doses also differed among the three groups, with a relative ratio of 1.0, 1.1 and 1.7 in the homEM, hetEM and PM groups, respectively. The mean AUC values for RPZ after a single and repeated doses were significantly different between the homEM and PM groups, but not between the homEM and hetEM, or hetEM and PM groups. No significant change in the mean AUC values for RPZ from a single dose to repeated doses was observed in any of the three genotype groups.

CYP2C19 genotype and 24-h intragastric pH

The median intragastric pH value of the PM group was the highest, followed by that of the hetEM group, with that of the homEM group being the lowest (Table 2). No significant differences in intragastric pH values were observed among the three groups after a single dose and repeated doses of RPZ for 8 d. No significant changes in intragastric pH values from single to repeated doses were observed in the three genotype groups. The ratio of intragastric pH median after a single dose and that after repeated doses ranged from 84.5% to 107.4%, indicating that the metabolism of RPZ after a single dose could

Table 2 Median (interquartile range) 24 h pH after a single and repeated doses of RPZ 20 mg

	HomEM	HetEM	PM
	3.82	4.36	6.09
D 1	(2.1-5.2)	(2.0-6.1)	(4.9-6.8)
	4.52	4.37	5.67
D 8	(3.0-5.5)	(2.9-6.0)	(4.8-6.5)
D 1/D 8 (%)	84.5	99.8	107.4

Table 3 Serum gastrin level with reference to CYP2C19 polymorphism

	HomEM		HetEM		PM	
	D 1	D 8	D 1	D 8	D 1	D 8
Gastrin AUC (pg/mL per h) ^a	812.03 ± 147.02	1169.98 ± 333.70	964.08 ± 377.20	1771.38 ± 1024.90	1181.06 ± 420.70	1897.45 ± 1359.59

^aP < 0.05, AUC on d 1 vs AUC on d 8.

attain maximum acid-inhibitory efficacy.

CYP2C19 genotype and serum gastrin level

The 24-h mean serum gastrin concentration-time profiles are given in Figure 1. The mean serum gastrin concentration in PMs was significantly higher during all time periods than that in EMs. The mean serum gastrin AUC values in hetEMs observed after a single and repeated doses were between that of homEMs and PMs. The mean gastrin AUC values differed between the three genotype groups, with a relative ratio of 1.0, 1.2 and 1.5 after a single dose and 1.0, 1.5 and 1.6 after repeated doses in the homEM, hetEM and PM groups, respectively (Table 3). The gastrin AUC values among the three genotype groups showed no significant differences after a single dose and repeated doses of RPZ 20 mg. Significant increments in gastrin AUC values from a single dose to repeated doses were observed in the three different genotype groups.

As shown in Tables 1, 2 and 3, the subjects who had the lowest intragastric acidity showed the highest serum gastrin levels and the concentrations of RPZ.

DISCUSSION

PPIs, such as omeprazole and rabeprazole, have been used widely in the treatment of acid-related diseases. Studies have found that CYP2C19 is a major enzyme for the metabolism of PPIs in the liver^[12], and the inhibitory effects of PPIs are associated with the genotype of CYP2C19. The *in-vitro* human liver microsomal and *in-vivo* human pharmacology studies have shown that the metabolic profile of RPZ differs somewhat from other PPIs. RPZ is metabolized mainly *via* a nonenzymatic reduction to rabeprazole thioether^[12-14], and CYP2C19 and CYP3A4 are partially involved in the metabolism of RPZ^[12-14]. In addition, it has been shown that RPZ has a more rapid and powerful onset of pharmacological action^[15]. Yasuda^[10] found that the AUC for RPZ is not significantly increased after repeated doses. Our results showed that the AUC for RPZ after a single dose exceeded 80 percent of that after

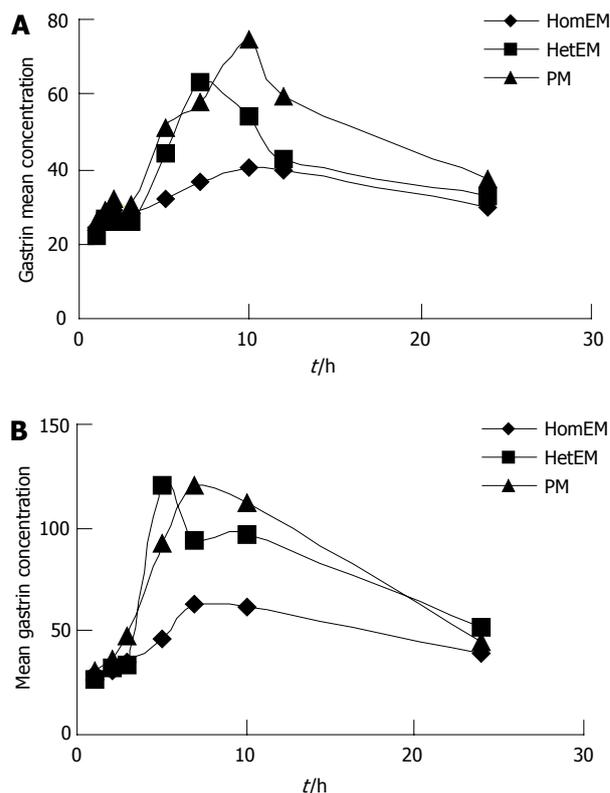


Figure 1 Mean serum gastrin concentration-time curve after a single dose (A) and repeated doses (B) of rabeprazole 20 mg.

repeated doses, and the gastric pH values after a single dose reached 84.5 to 107.4 percent of those after repeated doses. These findings suggest that the human body could absorb RPZ well, and RPZ could attain better acid-inhibitory efficacy.

Horai^[16,17] reported that CYP2C19 genotypic differences affect metabolism and pharmacokinetics of RPZ, and influence the gastric pH values and gastrin level in plasma. However, the majority of researchers^[3-6] believe that the acid-inhibitory efficacy and metabolism of RPZ are not dependent on the CYP2C19 genotype status. In our study, the acid-inhibitory efficacy and the gastrin level of rabeprazole were not influenced. This is supposed to be related with the small sample size of our study, as the number of subjects enrolled in each group was small. Ieiri^[18] reported that the mean AUC values for rabeprazole differed among the three genotype groups, with a relative ratio of 1.0, 1.7 and 3.8 in the homEM, hetEM and PM groups, being significantly related with genotype status. However, we found the AUC for RPZ was different markedly only between homEMs and PMs. Moreover, the intragastric pH, the best or most direct pharmacological index when using PPIs, had no significant differences among the three genotype groups after a single dose. After repeated doses of RPZ, the intragastric pH and AUC for RPZ were not affected by the CYP2C19 genotype status either. As for the discrepancy between the pharmacokinetics and pharmacodynamics of RPZ, we hypothesized that the acid inhibitory effect of RPZ was powerful and rapid, even in the homEM group.

When a PPI inhibits acid secretion, plasma or serum gastrin levels will be increased according to the degree

of acid inhibition, and serum gastrin concentration correlates well with gastric acid suppression^[19]. Therefore, plasma or serum gastrin concentration could be viewed as an indirect marker of the pharmacodynamic effects^[20]. Ieiri^[18] reported that the intergenotypic difference in the gastrin AUC was seen after the first dose of RPZ, and the AUC of serum gastrin on d 8 differed significantly between homEMs and PMs. However, in our study, no significant difference in the mean gastrin AUC was observed among the three genotype groups on d 1 and d 8, nor were significant differences in the median gastric pH levels found. We presume that the intergenotypic difference in serum gastrin AUC was synchronized with that in median gastric pH, which are consistent with the findings by Furuta^[8]. Previous studies indicated that serum gastrin concentrations returned to normal shortly after the antisecretory treatment with RPZ was discontinued^[21,22]; however, this phenomenon seems to be in conflict with the longlasting action of PPIs^[23]. Therefore, serum gastrin concentrations might not accurately reflect PPI-induced changes in intragastric pH, when PPIs were used in the different CYP2C19 genotype groups.

In conclusion, our study investigated mainly the pharmacodynamic and pharmacokinetic effect of rabeprazole on serum gastrin levels in association with different CYP2C19 genotypes. The acid-inhibitory effects of RPZ are independent of the CYP2C19 genotype status as well as the pharmacokinetic characteristics in the human body. Although the median gastric pH by RPZ treatment is related with the serum gastrin level, there is no significant difference between the different CYP2C19 genotype groups. RPZ is not only an effective proton pump inhibitor for treating the acid-related disease, but also does not affect serum gastrin level in association with CYP2C19 genotype status.

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CASE REPORT

Gastric sarcoidosis mimicking irritable bowel syndrome-Cause not association?

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Abstract

Sarcoidosis is a systemic disease of unknown aetiology that may affect any organ in the body. The gastrointestinal tract however is only rarely affected outside the liver. Symptoms may be non-specific. Irritable bowel syndrome (IBS) is a common diagnosis. The recognition of IBS is aided by the use of the Rome II criteria - in the absence of organic disease. We describe the first case of a patient with gastric sarcoidosis who presented with IBS symptoms but subsequently responded to immunosuppressive therapy.

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Key words: Gastrointestinal; Sarcoidosis; Irritable bowel syndrome

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<http://www.wjgnet.com/1007-9327/12/4754.asp>

INTRODUCTION

Sarcoidosis is a systemic disease of unknown aetiology characterised by the presence of non-caseating epithelioid granulomas. The lungs, eyes and skin are the most commonly affected organs^[1]. Liver involvement is well recognised but symptomatic involvement of the gastrointestinal (GI) tract occurs in less than 1% of

cases^[2-6]. Presenting GI symptoms in order of frequency are abdominal pain, haematemesis, nausea and vomiting and weight loss^[1,2,4-6]. This observation is only based on the case reports and case series that have been reported^[2,4-6]. Irritable bowel syndrome (IBS) is a common diagnosis and the symptom based Rome II criteria may be used to support the diagnosis^[7]. However it is essential when investigating patients who present with IBS type symptoms that organic disease is excluded^[7]. We describe the first case of a patient with gastric sarcoidosis who presented with IBS symptoms but subsequently responded to immunosuppressive therapy.

CASE REPORT

A 27 year old Caucasian man presented to the respiratory physicians with chest discomfort and 'flu-like symptoms' in 1983. A plain radiograph of the chest showed bilateral hilar lymphadenopathy and left middle zone infiltrates. Gallium scanning and a positive Kveim test confirmed the diagnosis of pulmonary sarcoidosis. Remission was achieved with oral steroids. Apart from a chronic irritable cough, he had no recurrence of his respiratory symptoms over the following 22 years. His previous annual serum angiotensin converting enzyme (ACE) and a recent high resolution computed tomography (CT) of the chest was normal.

He presented to a gastroenterology department with a 6-mo history of upper/central abdominal pain relieved on defaecation. There was a change in frequency of bowel movement - he now opened his bowels less than three times per week (prior to this presentation he opened his bowels daily). The stool varied between normal and pellet-like. He described bloating, distension and tenesmus. There was no weight loss or any other sinister symptoms. His symptoms fulfilled the Rome II criteria for constipation predominant IBS. His inflammatory markers (ESR and CRP) were normal. Further investigations including an upper GI endoscopy, colonoscopy, ultrasound and CT of the abdomen were all normal. The diagnosis of IBS was suggested but the patient still had concerns about the cause of his symptoms. With the consent of the clinicians from the initial centre the patient sought a second opinion. Although he had no pulmonary symptoms, (apart from a chronic cough) we wondered if his IBS symptoms could be due to gastrointestinal sarcoidosis. Further investigations in our centre showed a raised serum ACE level of 67 IU/mL (Range 18-55 IU/mL). In addition,



Figure 1 Gastric antrum viewed from mid body of stomach showing widespread erythematous changes consistent with gastritis.

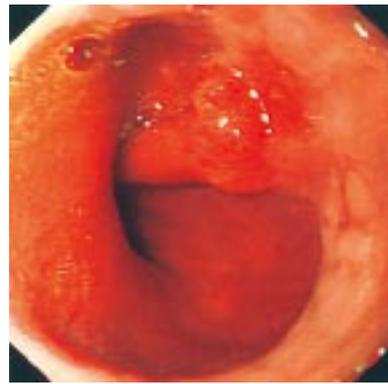


Figure 2 Gastro-oesophageal junction showing polypoid lesion.

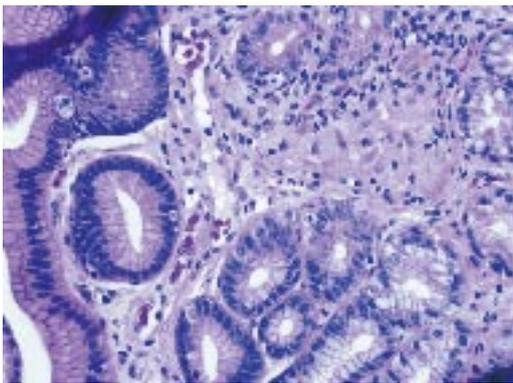


Figure 3 Gastric antral biopsy (HE x 400 magnification) showing surface epithelium on the left of the image. The glands are displaced by a central granuloma formed from loosely aggregated epithelioid histiocytes, with a few surrounding lymphocytes.

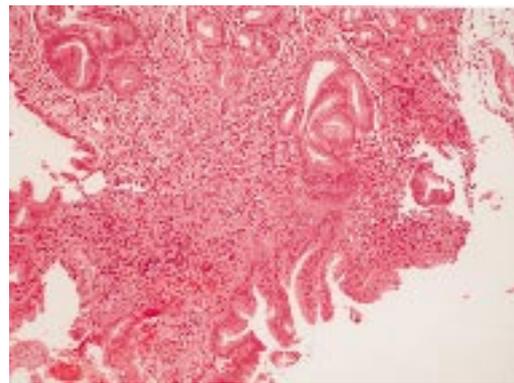


Figure 4 Biopsy of polypoid lesion at gastro-oesophageal junction showing glandular tissue with active chronic inflammation and reactive epithelial changes (HE x 200 magnification).

he also had a positive IgG antigliadin antibody and a serum IgA of 0.52 G/L (0.8-4.0 G/L) but negative IgA antigliadin and anti-endomysial antibody (EMA). We performed a gastroscopy, which showed antral erythema (Figure 1) and a polypoid lesion at the gastro-oesophageal junction (Figure 2). Duodenal biopsies were normal. Biopsy of the antrum revealed granulomas consistent with sarcoidosis (Figure 3). Biopsy of the polypoid lesion showed chronic inflammatory cell infiltrate only (Figure 4). His CLO test was negative and there was no evidence of *H pylori* or alcohol-acid fast bacilli in any of the biopsy specimens.

Oral prednisolone initially achieved some symptomatic benefit, but he responded subjectively to a short course of intravenous hydrocortisone. During this time his serum ACE normalised and he was discharged on 30 mg prednisolone. He has subsequently had an octreotide scan showing no activity in the stomach (whilst on prednisolone) and was started on oral azathioprine with progressive resolution of his GI symptoms.

DISCUSSION

Sarcoidosis of the GI tract is rare but when involved the stomach is the commonest site. Gastric granulomas have coincidentally been reported in up to 10% of patients with pulmonary sarcoidosis^[8,9]. However, whether the presence of gastrointestinal sarcoid accounts for GI

symptoms is controversial. Previous investigators have described endoscopic and radiological findings of peptic ulceration, inflammation and gastric outlet obstruction^[2,3,5,10-12]. Diagnosis is achieved by biopsy confirming the presence of granulomas without evidence of any other granulomatous disorders. One series of 67 patients with sarcoidosis described 7.5% of patients as having abdominal pains at diagnosis. Further evaluation revealed 15% had liver involvement (biopsy proven) and 36% had luminal GI disease; reflux oesophagitis (21%), IBS (10%) and inflammatory bowel disease (4.5%)^[13]. Both reflux and irritable bowel syndrome symptoms are frequent amongst the general population, however, this study suggests that the presence of such symptoms may require investigation. The optimal approach would be upper GI endoscopy and biopsy of any abnormal areas.

This is the first reported case of a patient who fulfilled the Rome II criteria for IBS but was subsequently found to have gastric sarcoidosis. Although we performed antigliadin antibodies (and EMA) to exclude coeliac disease-IgG antigliadin lacks specificity and has previously been reported in association with gastrointestinal sarcoidosis^[14,15]. If we had not performed antral biopsies having seen macroscopic evidence of inflammation, then the diagnosis may have been missed. It is possible that in our case both gastric sarcoid and IBS co-exist. We believe that this is unlikely given the symptomatic and serological response to immunosuppression^[16,17]. In addition, a normal

octreotide scan whilst on prednisolone (with no areas of increased uptake) also suggests remission and further corroborates our view^[16].

In conclusion, we report the first case of gastric sarcoid presenting with Rome II criteria for IBS. Our case suggests that patients with suspected functional GI symptoms in the presence of pulmonary sarcoid, may have sarcoidosis of the GI tract. It is imperative that the endoscopist is aware of this possibility and performs a biopsy of any macroscopically abnormal areas. By taking this approach patients with symptomatic GI sarcoid may benefit from early diagnosis and symptomatic relief with prednisolone or immunosuppressive therapy.

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Sustained viral response of a case of acute hepatitis C virus infection via needle-stick injury

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Abstract

A 29-year-old nurse with a hepatitis C virus (HCV) infection caused by needle-stick injury was treated with interferon-beta starting about one year after the onset of acute hepatitis. The patient developed acute hepatitis C with symptoms of general fatigues, jaundice, and ascites 4 wk after the needle-stick injury. When these symptoms were presented, the patient was pregnant by artificial insemination. She hoped to continue her pregnancy. After delivery, biochemical liver enzyme returned to normal levels. Nevertheless, HCV RNA was positive and the pathological finding indicated a progression to chronicity. The genotype was 1b with low viral load. Daily intravenous injection of interferon-beta at the dosage of six million units was started and continued for eight weeks. HCV was eradicated without severe adverse effects. In acute hepatitis C, delaying therapy is considered to reduce the efficacy but interferon-beta therapy is one of the useful treatments for hepatitis C infection in chronic phase.

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Key words: Hepatitis C virus ; Acute hepatitis; Interferon-beta; Needle stick injury

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INTRODUCTION

Needle-stick injury is a major cause of acute hepatitis C in health care workers^[1]. Acute infection of hepatitis C virus (HCV) frequently progresses to chronic hepatitis, which leads to liver cirrhosis and to hepatocellular carcinoma^[2]. Preventing the progress to chronicity is necessary, and several treatment strategies of interferon therapies were reported^[3,4,5,6]. Early phase treatment is recommended in order to obtain a sustained virological response (SVR)^[5]. On the other hand, optimal timing of treatment is controversial because self-limited hepatitis exists in 15%-30% of acute hepatitis C^[7]. Several predictive factors of the spontaneous clearance of HCV were reported but these have not been confirmed in larger numbers and well-designed prospective studies. As wait & see strategy to expect the spontaneous viral clearance, it was reported that 8 to 12 wk of delaying therapies obtained more than 90% of SVR rate, however, a delay by one year reduced the rate^[7,8]. We report a case of acute hepatitis C infection caused by needle-stick injury just before pregnancy. After delivery, the patient achieved a SVR by treating with interferon-beta in the chronic phase of acute hepatitis C.

CASE REPORT

Patient

The patient was a 29-year old Japanese woman. She was a clinical nurse. She had a missed abortion at the age of 26. She admitted occasional alcohol intake but she did not receive blood transfusions or operations.

Present illness

She encountered a skin puncture on the hand from a needle contaminated with blood from a patient with chronic hepatitis C on September 15th, 2001. She became pregnant through artificial insemination on October 2nd. She felt general fatigue from October 16th. Her attending physician pointed out the abnormal liver function tests, and then she was referred to Sendai City Medical Center at October 19th. On admission, liver function tests indicated elevated aspartate aminotransferase (AST; 549 IU/L, normal 12-30 IU/L) and alanine aminotransferase (ALT; 870 IU/L, normal 8-35 IU/L) activities. Total bilirubin (1.0 mg/dL, normal 0.2-1.2 mg/dL), albumin (39 g/L, normal 42-53 g/L), and prothrombin time (PT; 93%)

Table 1 Laboratory findings at October 19th

WBC	6530 / μ L	T-Bil	1.0 mg/dL	HCV-Ab	positive
Hb	13.0 g/ μ L	D-Bil	0.7 mg/dL	HCV-RNA	> 850 kIU/L
Plt	200 $\times 10^3$ / μ L	Total protein	71 g/L	HCV genotype	1b
PT	93%	Albumin	39 g/L	HAV IgM	negative
AST	549 IU/L	BUN	6.4 mg/dL	HBs-Ag	negative
ALT	870 IU/L	Creatinine	0.5 mg/dL	HBs-Ab	negative
LDH	497 IU/L	Na	140 mEq/L	IgM Hbc-Ab	negative
ALP	181 IU/L	K	4.0 mEq/L	EBV IgM	negative
γ -GTP	259 IU/L	Cl	106 mEq/L	HSV IgM	negative
ChE	94 IU/l	Glucose	92 mg/dL	CMV IgM	negative

WBC: white blood cell; Hb: hemoglobin; Plt: platelet; PT: prothrombin time; AST: aspartate aminotransferase; ALT: alanine aminotransferase; LDH: lactate dehydrogenase; ALP: alkaline phosphatase; γ -GTP: gamma glutamyl transpeptidase; ChE: choline esterase; T-Bil: total bilirubin; D-Bil: direct bilirubin; BUN: blood urea nitrogen; HCV: hepatitis C virus; HAV: hepatitis A virus; HBs-Ag: hepatitis B virus surface antigen; Hbc-Ab: hepatitis B virus core antibody; EBV: Epstein-Barr virus; HSV: herpes simplex virus; CMV: cytomegalovirus.

indicated that hepatic functional reserve was maintained (Table 1). Anti-HCV antibody which was negative before the pregnancy, converted to positive, and HCV RNA test revealed high viral load with genotype 1b. Therefore, she was diagnosed with acute hepatitis C. A small amount of ascites was detected on the surface of the liver by ultrasound imaging.

She hoped for continuation of the pregnancy, thus, conservative medical management was performed and liver function tests improved (Figure 1). After that, HCV RNA decreased below detection sensitivity of the routine quantitative method (Cobas Amplicor HCV Monitor test, Roche Diagnostic Systems, Branchburg, NY, USA) although remaining positive with qualitative method (Cobas Amplicor HCV test, Roche). On June 17th, 2002, she delivered safely. Liver function tests were within normal ranges but HCV RNA still remained positive. At this point, spontaneous viral clearance was not expected, thus, she was admitted to our hospital to receive anti-viral therapy on September 9th 2002.

Physical findings at administration

Physical examination revealed: height 158 cm; weight 54 kg; blood pressure 118/64 mmHg; body temperature 36.3°C; and clear consciousness. The bulber conjunctiva was not icteric. No peripheral edema, vascular spider, and flapping tremor were observed.

Laboratory findings

The result demonstrated normal liver function, no evidence of coagulopathy, anemia, or thrombocytopenia. HCV RNA indicated 1.0 kIU/L, which was under detection sensitivity previously.

Pathology

A liver biopsy specimen taken before anti-viral therapy demonstrated chronic hepatitis with mild activity without fibrosis. Swollen Kupffer cells were seen in diastase-periodic acid-Schiff preparations (Figure 2).

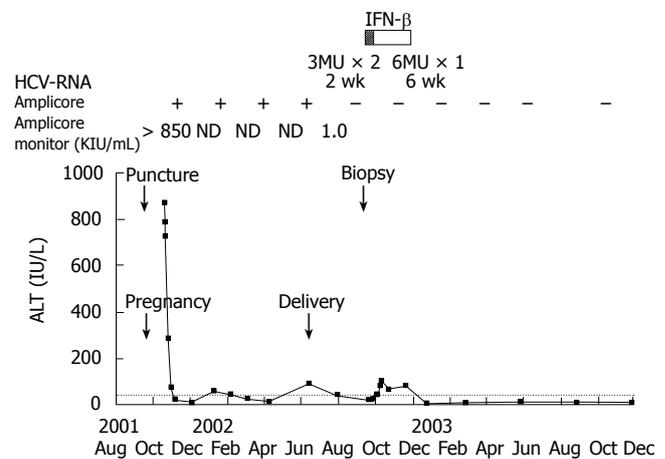


Figure 1 Clinical course of an acute hepatitis C patient receiving interferon-beta (IFN- β). MU, million units.

Clinical course

After informed consent was obtained, the patient received six million units of interferon-beta (Mochida Pharmaceutical Co., Ltd., Tokyo, Japan) by intravenous drug injection daily for eight weeks. She developed a daily fever after the initiation of treatment but recovered in a brief period of time. Other adverse effects were not seen during the treatment. HCV RNA became negative two weeks after the initiation of treatment (Figure 1). ALT increased in slight degree but improved after the end of the treatment (Figure 1). HCV RNA negativity and normalization of ALT level were sustained for 40 mo after the end of interferon treatment.

DISCUSSION

In Japan, 85% of hepatocellular carcinoma patients are associated with chronic HCV infection^[9]. Thus, to inhibiting the progression of acute HCV infection to chronicity is very important for the prevention of

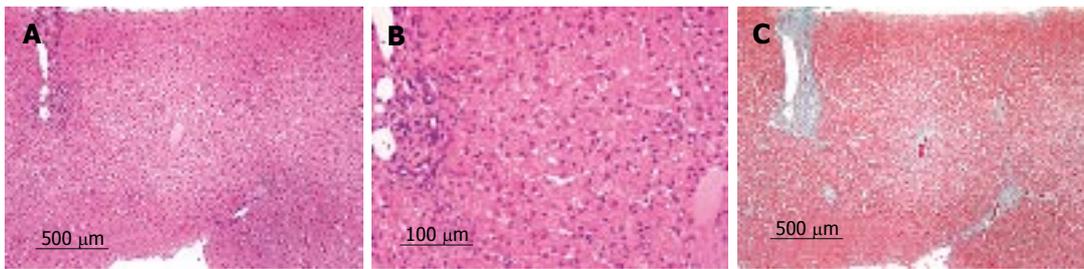


Figure 2 Microscopic pathological findings. **A:** (HE, x 4) and **B:** (HE, x 10) showed inflammatory change with mild activity; **C:** (Elastica-Masson staining, x 4) showed slight fibrotic change.

hepatocellular carcinoma. Recently, as the cause of post-transfusion hepatitis, HCV infection decreased to extremely low levels because of the establishment of diagnostic methods such as HCV-antibody or HCV RNA detection^[10]. However, acute hepatitis C still exists by needle-stick injury in health care workers^[11]. Interferon therapy has been indicated for acute hepatitis C for many years but the strategies were controversial in the points of optimal timing of starting therapy, treatment regimen, duration of interferon injection, and management of self-limited patients. Therefore the standard therapy for acute hepatitis C has not been established.

Treatment regimen varies with the times and institutes. Interferon-beta was indicated in the early 1990's^[11]. Interferon-alpha based therapies have been widely indicated, doses were 3-6 million units, durations were 24-52 wk, and recently, combination with ribavirin and pegylated-interferon were reported^[12].

Jaeckel *et al* reported that they treated acute hepatitis C with interferon-alpha (5 million units, thrice weekly for 20 wk) and obtained virological response in 98% of cases, thus, they suggested early treatment immediately on diagnosis^[5]. On the other hand, self-limited hepatitis was reported in 15%-30% of acute hepatitis C^[7]. Identifying the patients who recover spontaneously is important because interferon therapy involves considerable adverse effects, compromises patients' quality of life for long-term treatment, and is highly expensive. Predictable factors of self-limited acute hepatitis C were reported. Female gender^[6], younger age^[13], and the lack of complications such as human immunodeficiency virus infection^[14] were reported as host factors. HCV genotype 3 and 2 were reported as virological factors^[15]. Clinical features during the acute phase of infection as symptomatic disease^[6] and fast and continuous decline in viral load were reported with a lower rate of chronicity^[7]. Hofer *et al* reported that mean duration of spontaneous recovery from acute HCV infection was 77 d from infection and 35 d from the onset of symptoms^[7]. Gerlach *et al* reported that they treated acute hepatitis C patients who could not obtain spontaneous viral clearance within 12 wk by interferon-alpha based therapy (3-5 million units, thrice weekly for 14-61 wk) and that the overall virological response was 91% of cases^[6]. A meta-analysis indicated that delaying therapy by 60 d after the onset of symptoms did not reduce the efficacy of treatment^[16]. Wedemeyer *et al* defined the course of acute hepatitis C virus infection as five phases which were: phase A, post-exposure; phase B, asymptomatic viremic patients with normal ALT, two to six weeks post-exposure before the onset of symptoms;

phase C, acute hepatitis with ALT elevation and symptoms; phase D, spontaneous recovery or partial tolerization; phase E, chronic hepatitis after more than six months of infection^[17]. Nomura *et al* reported that the SVR to 24 wk of interferon-alpha was 100% when therapy was initiated in acute hepatitis phase, but fell to 53% if treatment was applied in chronic phase^[8], though it seems a much high rate to be compared with the SVR rate of conventional interferon monotherapy in chronic hepatitis C.

In our case, we initially expected spontaneous recovery because the patient was of female gender, younger age, because she developed symptoms of jaundice and ascites, and because a fast and continuous decline in viral load was observed. However, HCV RNA level remained at 1.0 kIU/L on year after the onset of the infection. Also liver biopsy indicated the progress to chronic hepatitis. The patient desired to complete the treatment in a short period of time because she has recently completed delivery and she wanted to spend the time with her child and to breastfeed the child, thus we treated her with interferon-beta for eight weeks and obtained the SVR.

Interferon-beta therapy is a useful treatment for acute hepatitis C in chronic phase and is superior to interferon-alpha based therapy with regard to treatment duration. Further studies are necessary to clarify the efficacy of this therapy for acute hepatitis C.

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Amyand's hernia: A case report

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Abstract

The presence of vermiform appendix in inguinal hernia is rare and is known as Amyand's hernia. We report an Amyand's hernia, where the appendix was found in a right inguinal hernia in one male cadaver aged ninety two years.

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Key words: Amyand's hernia; Appendix; Inguinal hernia

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INTRODUCTION

Inguinal hernia may display very unusual sac contents. Ovary, fallopian tube, urinary bladder, incarcerated bladder diverticula, large bowel diverticula with the form of diverticulitis or abscess, Meckel's diverticulum (Littre hernia) or foreign bodies (e.g., fishbones) have been rarely reported^[1-3]. The presence of the appendix within an inguinal hernia has been referred to as "Amyand's hernia" to honour Claudius Amyand, surgeon to King George II. Amyand was the first to describe the presence of a perforated appendix within the inguinal hernial sac of an eleven-year old boy and performed a successful transherniotomy appendectomy in 1735^[4,5].

CASE REPORT

In a male cadaver, 92 years old, at the time of death, a right inguinal hernia was recognized during a student descriptive anatomy laboratory. The medical history of the patient included arterial hypertension that had been treated for the past twenty years and the right inguinal hernia (without herniotomy) that had existed for the past ten years. The deep fascia, the external oblique aponeurosis and the spermatic cord were identified, as well as the hernial sac. Upon opening the hernial sac, the vermiform appendix was found free within, without adhesions to the sac (Figure 1, Figure 2, Figure 3). Macroscopically, the vermiform appendix was 8 cm in length with a maximum diameter of 0.9 cm. Histological examination did not reveal any pathological alterations (Figures 4A and B).

DISCUSSION

A hernia is defined as the protrusion of a viscus or part of a viscus through the walls of its containing cavity. The presence of the appendix within an inguinal hernial sac is referred to as "Amyand's hernia" and is an uncommon condition. The incidence of having a normal appendix within the hernial sac varies from 0.5% to 1%, whereas only 0.1% of all cases of appendicitis present in an inguinal hernia, underscoring the rarity of the condition^[6,7]. The majority of the reported cases present with the features of an obstructed or strangulated inguinal hernia. Even acute appendicitis or perforation of the appendix within the sac simulates perforation of the intestine within the hernia, and does not have specific symptoms or signs. Due to these facts it is very difficult to reach a clinical diagnosis of Amyand's hernia preoperatively. In fact, the diagnosis is made intraoperatively as the patient undergoes surgical exploration for a complicated inguinal hernia. A preoperative computed tomography scanning of the abdomen could be helpful for diagnosis, but this is not a routine practice after the clinical suspicion of a complicated inguinal hernia^[8]. However, one case of a three-month old boy has been reported in which a right-sided sliding appendiceal inguinal hernia was diagnosed preoperatively with sonography^[9].

The occurrence of herniated appendices is mostly reported in a right inguinal hernial sac, probably as a consequence of the normal anatomical position of the appendix and also because right sided inguinal hernias



Figure 1 Right inguinal hernial sac.



Figure 2 Inguinal hernia with vermiform appendix at the opening of the sac.



Figure 3 Appendix in inguinal hernia.

are more common than left sided hernias^[10]. An extensive literature search revealed three reported cases of left sided Amyand's hernia^[11-13]. The occurrence of left sided Amyand's hernia may be associated with the presence of a situs inversus or malrotation as an underlying cause. An abdominal computed tomography scan or X-ray examination should be performed to exclude these entities^[14].

Most of the published cases have been reported as appendicitis incarcerated in a hernia. It is difficult to determine whether a primary visceral inflammation, which could be referred to as appendicitis, is the pathological

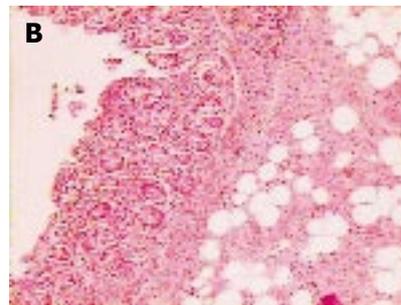
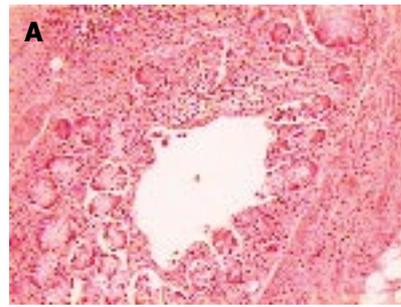


Figure 4 Pathological examination of the appendix found in inguinal hernia. A: HE x 100; B: HE x 400.

mechanism, or if the primary event is strangulation of the herniated appendix, leading subsequently to ischemic necrosis and secondary inflammation^[15]. The presence or absence of inflammation of the appendix is a very important determinant of appropriate treatment. If inflammation of the organ and incipient necrosis are present, a transherniotomy appendectomy should be performed. The presence of pus or perforation of the organ is also an absolute contraindication to the placement of a mesh for hernia repair. Associated intra-abdominal abscesses, if present, may be dealt with either percutaneously or by open drainage. The majority of the authors agree that a normal appendix within the hernial sac does not require appendectomy, and that every effort should be made to preserve the organ found in the hernia sac for an uneventful postoperative course^[16,17].

Finally, we conclude that the presence of the appendix in an inguinal hernial sac, referred to as "Amyand's hernia", is an uncommon entity. Despite its rarity, the fact that the majority of such cases present as a complicated inguinal hernia, making preoperative diagnosis difficult, demands that surgeons consider this condition in their differential diagnosis and so they are able to offer appropriate treatment.

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CASE REPORT

Mutations of the *AAAS* gene in an Indian family with Allgrove's syndrome

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Abstract

The triple A or Allgrove's syndrome is an autosomal recessive disorder characterized by the triad of achalasia cardia, alacrima and ACTH resistant adrenocortical insufficiency. Mutations of the Achalasia-Addisonianism-Alacrima-Syndrome (*AAAS*) gene on chromosome 12q13 are associated with this syndrome. We report an Indian family where two siblings were homozygous for a known mutation of the *AAAS* gene and presented with the classical triad of symptoms. The mother and the brother were heterozygous and asymptomatic. The affected siblings had iron deficiency anemia and the younger sister had pes cavus and palmoplantar keratosis. Neurological symptoms were absent in both affected children. Recognition of this syndrome can lead to early treatment of adrenal insufficiency and genetic counselling.

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Key words: Allgrove's syndrome; Triple A syndrome; Autosomal recessive; Genetic mutation; India

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INTRODUCTION

Allgrove's syndrome, also known as the triple A syndrome is characterized by the triad of adrenal insufficiency, achalasia cardia and alacrima^[1]. Ever since its first description in 1978, more than 70 cases have been reported in the literature. The condition is associated with impairment of the central, peripheral and autonomic nervous systems, suggesting that it is

a result of a mutation in a pleiotropic gene. Other reported abnormalities include palmoplantar hyperkeratosis, short stature, osteoporosis and microcephaly^[2-4]. Progression of neurological symptoms has been reported with worsening of peripheral neuropathy, dementia, long tract degeneration, dysarthria and ataxia^[5,6].

Pathological studies from esophageal myomectomy specimens of children afflicted with Allgrove's syndrome show fibrosis of the intermuscular plane and absent myenteric ganglia^[3]. Neuronal NO synthase has also been found to be deficient and could explain the poor relaxation of the lower esophageal sphincter (LES). Necropsy has been carried out in one of the pairs of original siblings published, which reveals atrophy of the adrenal cortical zona fasciculata and zona reticularis and absence of ganglion cells and nerve fibers in the lower esophagus^[1].

This syndrome has been mapped to a novel gene on chromosome 12q 13 near the type II keratin gene cluster, and the gene is designated as the Achalasia-Addisonianism-Alacrima-Syndrome gene (the *AAAS* gene)^[7]. There is a great deal of variability of symptoms and severity between patients with the same *AAAS* mutation, implying variable expression of this mutated gene.

This report describes the clinical presentation of two affected siblings with Allgrove's syndrome and analyses the mutations in the immediate family.

CASE REPORT

A seventeen year old young lady presented along with her fifteen year old younger sister with identical symptoms of dysphagia, excessive fatigue and skin pigmentation. The elder sibling had been symptomatic for eight years and her sister for seven years respectively. Both had progressive difficulty in swallowing and regurgitation of food at the time of presentation. They had extreme tiredness and fatigue. The mother had noticed progressive pigmentation in both her daughters. The younger sibling had several episodes of sporadic abdominal pain and vomiting. Their milestones had been normal and they had attained menarche and thelarche normally. Their parents were non-consanguineous and their father had expired following an accident. One elder sister had died of meningitis at nine years of age and on questioning the mother, she mentioned the daughter having similar facial hyperpigmentation. A brother was asymptomatic at 16 years of age. Examination of both patients revealed normal blood pressure and heart rate with no postural changes. Both sisters had facial and palmar hyperpigmentation. The younger sibling had bilateral

Table 1 Summary of clinical features and laboratory investigation in both siblings with Allgrove's syndrome

Variable	Elder sibling	Younger sibling
Dysphagia	Present	Present
Generalized pigmentation	Present	Present
Abdominal pain	Absent	Present
Decreased lacrimation	Present	Present
Postural hypotension	Absent	Absent
Palmoplantar keratosis	Absent	Present
Temporal optical pallor	Present	Present
Hemoglobin (mg%)	6.8	8.1
Albumin (mg%)	4.2	4.2
Pre-Synacthen cortisol ($\mu\text{g}/\text{deciliter}$)	3.94	3.37
Post-Synacthen cortisol ($\mu\text{g}/\text{deciliter}$)	5.16	4.1
Barium swallow	Aperistaltic dilated esophagus	Aperistaltic dilated esophagus
LES pressure on manometry (mmHg)	45	60

palmo-plantar keratosis and pes cavus. The conjunctiva and oral cavities of both sisters were dry. Schirmer's test confirmed decreased lacrimation. Both were noted to have bilateral temporal pallor of optic discs.

Hemogram showed iron deficiency anemia in both sisters. Biochemical tests and chest X ray were normal. Barium swallow showed dilated esophagus and absent peristalsis in both sisters. Esophageal manometry done to confirm the diagnosis showed elevated LES pressures (45 mm and 60 mm respectively), poor relaxation to swallow and absent body peristalsis consistent with achalasia. Serum 8 am cortisol was 3.94 $\mu\text{g}/\text{deciliter}$ and 3.37 $\mu\text{g}/\text{deciliter}$ in both sisters respectively and post Synacthen values were 5.16 $\mu\text{g}/\text{deciliter}$ and 4.10 $\mu\text{g}/\text{deciliter}$ respectively, consistent with Addison's disease. The clinical features and laboratory investigations are summarized in Table 1.

The confirmation of achalasia, Addison's disease, history of dry eye in both siblings, palmo-plantar hyperkeratosis in one sister and positive family history pointed to a clinical diagnosis of Allgrove's syndrome. Molecular testing for the *AAAS* gene causing Allgrove's syndrome was initiated to confirm the diagnosis. Blood samples from the patients, their mother and unaffected brother were taken after consent for *AAAS* gene analysis. Replacement therapy with corticosteroids and mineralocorticoids dramatically improved the symptoms in both the sisters. They have been advised to make frequent follow-up visits at the clinic.

MUTATION ANALYSIS

DNA was extracted using standard protocols. Coding sequences including exon-intron boundaries were amplified from genomic DNA and sequenced as reported previously^[8]. Sequencing of the coding regions and exon/intron junctions of the *AAAS* gene in the two sisters revealed a homozygous splice mutation in exon 1, resulting in a frameshift after glycine at amino acid position 14 and a premature stopcodon (G14fs). This is the result of the transversion C to A at nucleotide position 43, forming a new

premature splice donor site after G at nucleotide position 40 with a consecutive loss of the following 83 bp from exon 1. The mother is a heterozygote and so is the brother. Thus it verified the carrier status of the unaffected family members. This gene mutation analysis confirmed the diagnosis and helped in genetic counselling.

DISCUSSION

Triple A syndrome (Allgrove's syndrome) is an autosomal recessive genetic disorder occurring due to mutations in the *AAAS* gene, resulting in phenotypic manifestations of achalasia, addisonianism, alacrima and variable symptoms of progressive neurological involvement. Using positional cloning, the product of the *AAAS* gene, designated ALADIN for alacrima, achalasia, adrenal insufficiency neurological disorder, was identified as a protein belonging to the WD repeat family^[9]. Proteins belonging to this family have a wide functional diversity in that they are involved in signal transduction, RNA processing, vesicular trafficking, cytoskeleton assembly and cell division control^[10].

The ubiquitous expression of *AAAS* is in accordance with the multisystemic character of the disorder and the predominant occurrence in the adrenal gland, gastrointestinal and nervous structures is in line with the tissues mainly affected in triple A syndrome. Various mutations have been described in patients from European, North African, Asian and American populations^[8]. Ours is the first report of mutations in the *AAAS* gene in an Indian family with Allgrove's syndrome. The transversion of C to A at nucleotide position 43 results in a markedly truncated protein, and it has been reported and confirmed that the mutation caused the disease^[8].

Genotypic-phenotypic correlations have not been consistent in Allgrove's syndrome^[11]. We noted similarities in presentation in both these siblings. However, palmoplantar keratosis and pes cavus was present in only the younger sister despite both siblings having the same mutation. There are reports of patients presenting without hypoadrenalism, which is the hallmark of this syndrome^[12]. From a large series of patients it has been seen that frameshift, stop codon and functionally significant mutations are likely to lead to a more severe phenotype, most probably occurring by a loss-of-function effect of the mutated protein^[13]. Future studies need to focus on functional analysis of this mutated gene in order to comprehend the myriad presentations of the Allgrove's syndrome.

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TREM-1 expression during major abdominal surgery: Comment on the Gonzalez-Roldan *et al* paper

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TO THE EDITOR

I read with a great interest the paper by Gonzalez-Roldan *et al*^[1]. The authors reported on the pattern expression of TREM-1 during sepsis and major abdominal surgery as compared to healthy controls and concluded that TREM-1 expression increased on the surface of monocytes after surgery. Several points deserve consideration. First, no data related to TREM-1 expression on neutrophils is provided. Second, of the 7 surgical patients, only 4 had both pre- and post-surgery cytometry analysis. Among these 4 patients, 2 displayed a decrease of TREM-1 expression after surgery. Therefore, it seems quite hazardous, based on these data, to conclude that TREM-1 increases after major

uncomplicated surgery, reflecting a systemic inflammatory response.

The other question that arose from this study is related to the determination of the TREM-1 splice variant (svTREM-1). First, it is still unknown whether this variant is translated or not^[2]. Anyway, the soluble form of TREM-1 is not believed to be related to this variant^[3]. The authors observed an increase of the svTREM-1 mRNA after surgery or during sepsis. By using the described primers, one could also expect to see a PCR product corresponding to the natural form of TREM-1^[3]. Unfortunately, such a product is not reported. Finally, it is rather disappointing not to see any measurement of plasma sTREM-1 in these patients in order to better characterise the “inflammatory” patients and the septic ones^[4].

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Meetings

MAJOR MEETINGS COMING UP

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<<http://www.congre.co.jp/1st-aphba/>>

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Annual Scientific
20-25 October 2006
Las Vegas, NV

14th United European Gastroenterology Week, UEGW
21-25 October 2006
Berlin, Germany

APDW 2006: Asian Pacific Digestive Week 2006
26-29 November 2006
Lahug Cebu City, Philippines

EVENTS AND MEETINGS IN THE UPCOMING 6 MONTHS

Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
24-25 March 2006
Sydney - NSW
Falk Foundation e.V.
symposia@falkfoundation.de

10th International Congress of Obesity
3-8 September 2006
Sydney
Event Planners Australia
enquiries@ico2006.com
www.ico2006.com

Easl 2006 - the 41st annual
26-30 April 2006
Vienna, Austria
Kenes International

Prague hepatology 2006
14-16 September 2006
Prague
Foundation of the Czech Society of Hepatology
veronika.revicka@congressprague.cz
www.czech-hepatology.cz/phm2006

12th International Symposium on Viral Hepatitis and Liver Disease
1-5 July 2006
Paris
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isvhld2006@mci-group.com
www.isvhld2006.com

Falk Symposium 152: Intestinal Disease Part I, Endoscopy 2006 - Update and Live Demonstration
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symposia@falkfoundation.de

Falk Symposium 153: Intestinal Disease Part II, Immunoregulation in Inflammatory Bowel Disease - Current Understanding and Innovation
6-7 May 2006
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ILTS 12th Annual International Congress
3-6 May 2006
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www.its.org

Internal Medicine: Gastroenterology
22 July 2006-1 August 2006
Amsterdam
Continuing Education Inc
jbarnhart@continuingeducation.net

6th Annual Gastroenterology And Hepatology
15-18 March 2006
Rio Grande
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www.hopkinscme.net

World Congress on Gastrointestinal Cancer
28 June 2006-1 July 2006
Barcelona, Spain
c.chase@imedex.com

International Conference on Surgical Infections, ICSI2006
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European Society of Clinical Microbiology and Infectious Diseases
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www.icsi2006.se/9/23312.asp

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www.sages.org

Digestive Disease Week 2006
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Los Angeles
www.ddw.org

Annual Postgraduate Course
25-26 May 2006
Los Angeles, CA
American Society of Gastrointestinal Endoscopy
www.asge.org/education

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3-7 June 2006
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Current and future applications of *in vitro* magnetic resonance spectroscopy in hepatobiliary disease

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Abstract

Nuclear magnetic resonance spectroscopy allows the study of cellular biochemistry and metabolism, both in the whole body *in vivo* and at higher magnetic field strengths *in vitro*. Since the technique is non-invasive and non-selective, magnetic resonance spectroscopy methodologies have been widely applied in biochemistry and medicine. *In vitro* magnetic resonance spectroscopy studies of cells, body fluids and tissues have been used in medical biochemistry to investigate pathophysiological processes and more recently, the technique has been used by physicians to determine disease abnormalities *in vivo*. This highlighted topic illustrates the potential of *in vitro* magnetic resonance spectroscopy in studying the hepatobiliary system. The role of *in vitro* proton and phosphorus magnetic resonance spectroscopy in the study of malignant and non-malignant liver disease and bile composition studies are discussed, particularly with reference to correlative *in vivo* whole-body magnetic resonance spectroscopy applications. In summary, magnetic resonance spectroscopy techniques can provide non-invasive biochemical information on disease severity and pointers to underlying pathophysiological processes. Magnetic resonance spectroscopy holds potential promise as a screening tool for disease biomarkers, as well as assessing therapeutic response.

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Key words: Magnetic resonance spectroscopy; Liver; Hepatobiliary disease; Membrane metabolism

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INTRODUCTION

The nuclear magnetic resonance (NMR) phenomenon was first reported in 1946^[1] and has since been widely used by the scientific and medical communities. The 2003 Nobel Prize for Medicine was jointly awarded to Professor Sir Peter Mansfield and Professor Paul Lauterbur for their work on clinical NMR, and this serves to underline the impact on the medical community of the utility of this technique with all its diverse applications (www.nobel.se/medicine/laureates). In this highlight article, we explain the background to NMR and discuss various clinical studies that are relevant to the liver and the biliary system, with particular emphasis on *in vitro* applications that have analyzed body fluids, such as bile, or tissue obtained from liver biopsies.

NMR is a non-invasive and non-selective technique, allowing the study of molecular composition and structure. NMR forms the basis of magnetic resonance imaging (MRI) methodologies, which have been developed using whole body magnets. Spatial localization using magnetic field gradients has enabled very detailed images of the human body to be obtained. *In vivo* NMR spectroscopy studies can be performed as an adjunct to clinical MRI, as part of the same examination. Longitudinal studies can be readily undertaken using both of these MR applications. The term magnetic resonance spectroscopy (MRS) is used in this highlight topic to denote *in vivo* and *ex vivo* clinical NMR spectroscopy studies, and also encompasses *in vitro* NMR spectroscopy, performed in the laboratory at very high magnet field strengths.

AN OVERVIEW OF MR SPECTROSCOPY

Although a detailed knowledge of the physical principles that govern NMR is not essential for appreciating the scope of NMR applications, it is often helpful to have some understanding of some of the basic concepts that underlie these methodologies. The NMR technique

exploits the behavior of atomic nuclei in an externally applied magnetic field. Magnetic resonance occurs because of the quantum mechanical property of “spin”, which is intrinsic to certain atomic nuclei. Examples of such nuclei of clinical relevance include hydrogen-1 (^1H), carbon-13 (^{13}C), fluorine-19 (^{19}F), phosphorus-31 (^{31}P) and chlorine-35 (^{35}Cl). Such nuclei can be imagined to act like small bar magnets in a magnetic field by ‘lining up’ with or against the field, and can be excited by irradiation with non-ionizing radiofrequency (rf) energy. During relaxation following excitation, rf signals are generated which contain information regarding the magnetic environment experienced by each nucleus, and therefore about the molecules in which they exist. The resulting signals, the “free induction decay”, are detected by a receiver coil and can be expressed as a frequency spectrum by the mathematical process of Fourier transformation. The relative frequency position of a metabolite signal, its chemical shift, is dependent on the locally experienced magnetic field and therefore the local chemical environment. Consequently, each nucleus type within a molecule has a characteristic chemical shift. Hydrogen-1 (^1H) and phosphorus-31 (^{31}P) are the two nuclei most commonly used for biological studies, as they are ubiquitous in nature ($^1\text{H} = 99.985\%$, $^{31}\text{P} = 100\%$ natural abundance).

Metabolic significance of clinical MR spectroscopy

Jacobson and colleagues^[2] used MRS to examine the effects of hydration of DNA in 1954. Current investigations include both *in vitro* MRS studies on tissue samples or body fluids and *in vivo* whole-body MRS studies.

In vitro MRS studies include the analysis of body fluids (such as plasma, urine or bile), extracts of tissue, or small biopsy-sized specimens of intact tissue. *In vivo* MRS is more difficult to perform and is characterized by poorer resolution of metabolites than *in vitro* MRS. This is due to factors such as the lower magnetic field strengths used for *in vivo* MRS clinical studies and also to the effects of magnetic susceptibility and patient motion. The typical magnetic field strength is 1.5-3.0 Tesla (T) for clinical MRS studies and 11.7-18.8 T for *in vitro* MRS studies.

In vitro MRS can detect and characterize a range of metabolic components simultaneously, even if their chemical identities are unknown at the time of analysis. *In vitro* MRS studies therefore provide a comprehensive metabolic profile of the low molecular weight components in biofluids and tissues, reflecting levels of endogenous metabolites involved in key cellular pathways, which indicate the physiological and pathophysiological status. The technique can also provide a profile of exogenous agents, including xenobiotics and their metabolites, and give an indication as to their effects on endogenous compounds^[3]. For such reasons, *in vitro* MRS has become one of the most successful and popular techniques for biofluid analysis over the past 10 years^[4].

It is relevant to consider *in vitro* MRS applications to the liver in the context of metabolite findings from both *in vivo* ^{31}P and ^1H MRS studies of patients with liver disease, who have been examined using whole body MRI/MRS

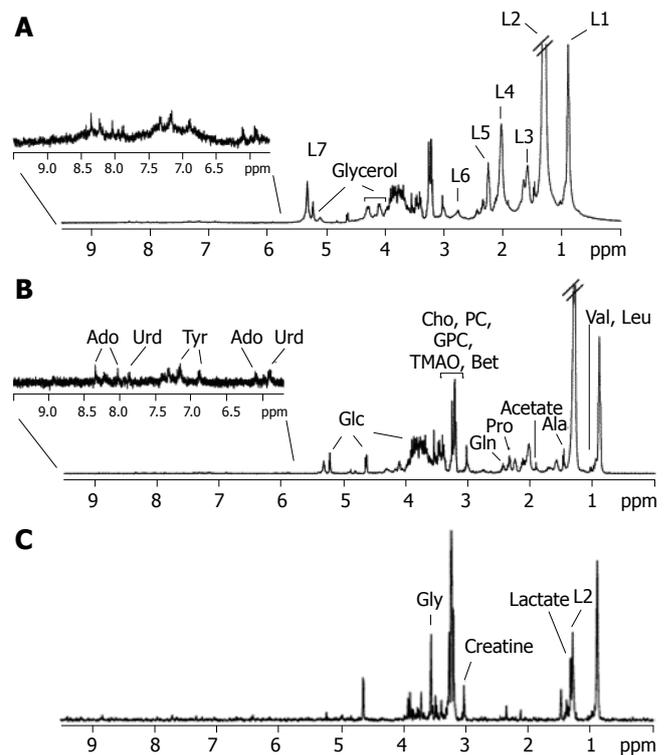


Figure 1 400-MHz ^1H HR-MAS NMR spectra of a human liver biopsy sample (rotation rate 4 kHz). **A:** Standard 1D spectrum; **B:** Spin-echo (CPMG) spectrum; **C:** JRES f2 projection. L1: lipid CH_3 ; L2: lipid $(\text{CH}_2)_n$; L3: lipid $\text{CH}_2\text{CH}_2\text{CO}$; L4: lipid $\text{CH}_2\text{-CH=CH}$; L5: lipid CH_2CO ; L6: lipid $\text{CH=CH-CH}_2\text{-CH=CH}$; L7: lipid CH=CH ; Cho: choline; PC: phosphocholine; GPC: glycerophosphocholine; TMAO: trimethylamine-N-oxide; Bet: betaine; Glc: glucose; Val: valine; Leu: leucine; Ala: alanine; Gln: glutamine; Gly: glycine; Tyr: tyrosine; Urd: uridine; Ado: adenosine. Reprinted with permission from Duarte *et al* Anal Chem 2005; 77: 5570-5578. Copyright (2005) American Chemical Society.

techniques. *In vivo* hepatic MRS studies have been primarily used in the research environment and have generally utilized ^{31}P MRS^[5], because ^1H MRS is technically more challenging^[6,7]. *In vivo* ^{31}P MRS gives information on cell turnover and energy state and has been used to grade patients with chronic liver disease^[8] and to aid in diagnosis and treatment response in patients with cancer^[9]. On the other hand, *in vivo* ^1H MRS allows quantification of intra-hepatocellular lipid (IHCL) levels, and therefore has been utilized recently to quantify the extent of steatosis in patients with fatty liver^[10,11].

Representative hepatic ^1H and ^{31}P MR spectra are illustrated in Figures 1-4. *In vitro* ^1H and ^{31}P MR spectra of liver tissue are illustrated in Figures 1 and 3, and these can be compared and contrasted with *in vivo* hepatic ^1H and ^{31}P MR spectra illustrated in Figures 2 and 4. An *in vitro* ^1H MR spectrum is dominated by contributions from water and IHCL. If the signal from water is suppressed using specific NMR methodologies, then more detailed contributions from IHCL can be observed along with resonances from choline-containing compounds (Cho) (Figures 1A-C). These data compare with the *in vivo* hepatic ^1H MRS spectrum, which allows quantification of water and IHCL (Figure 2B).

In vitro ^{31}P MR spectroscopy of liver tissue (Figure 3) allows characterization of resonances from phospholipid

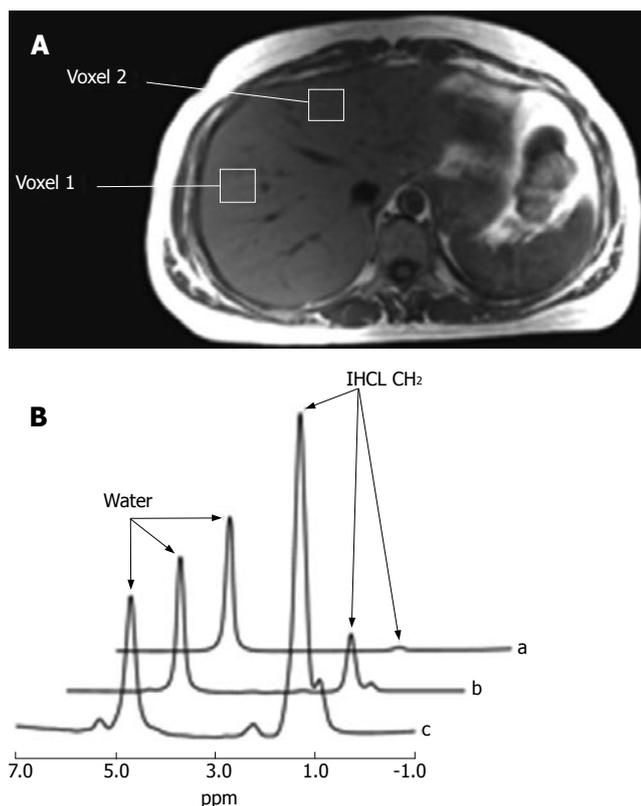


Figure 2 **A:** A transverse image through the abdomen showing the two voxel positions used to study regional variation in hepatic fat content; **B:** Typical proton magnetic resonance liver spectra from three volunteers showing progressive degrees of fatty infiltration. Spectrum (a) shows a liver with minimal fatty infiltration (1.0%), spectrum (b) shows a liver with moderate fatty infiltration (10.2%), and spectrum (c) shows a liver with severe fatty infiltration (74.9%). Resonances from water and IHCL-(CH₂)_n can be clearly identified. Values refer to the peak area of the IHCL peak with reference to the water peak after correcting for T₁ and T₂. IHCL: intrahepatocellular lipids. Reproduced from Thomas *et al* Gut 2005; 54: 122-127, with permission from the BMJ Publishing Group.

cell membrane precursors, including phosphocholine (PC) and phosphoethanolamine (PE), adenosine monophosphate (AMP) and glycolytic intermediates, such as glucose-6-phosphate. Cell membrane degradation products, including glycerophosphorylcholine (GPC) and glycerophosphorylethanolamine (GPE) and mobile phospholipids from the endoplasmic reticulum can also be identified. Depending on how the tissue is collected it is also possible to quantify contributions to the spectrum from inorganic phosphate and various nucleoside triphosphates (NTP). Such spectral resolution is difficult to achieve *in vivo*, and therefore a typical *in vivo* hepatic ³¹P MR spectrum (Figure 4) consists of six dominant, composite resonances, arising from: (1) the phosphomonoester region (PME), which are mainly cell membrane precursors and sugar phosphates; (2) inorganic phosphate (Pi); (3) phosphodiester (PDE), which are mainly cell membrane degradation products, but also signal from mobile phospholipids contained in mitochondria and (4-6) three resonances from NTP, which are chiefly composed of adenosine triphosphate (ATP), but contain contributions from uridine triphosphate, guanosine triphosphate and cytosine triphosphate^[12].

Changes in the PME and PDE metabolites indicate

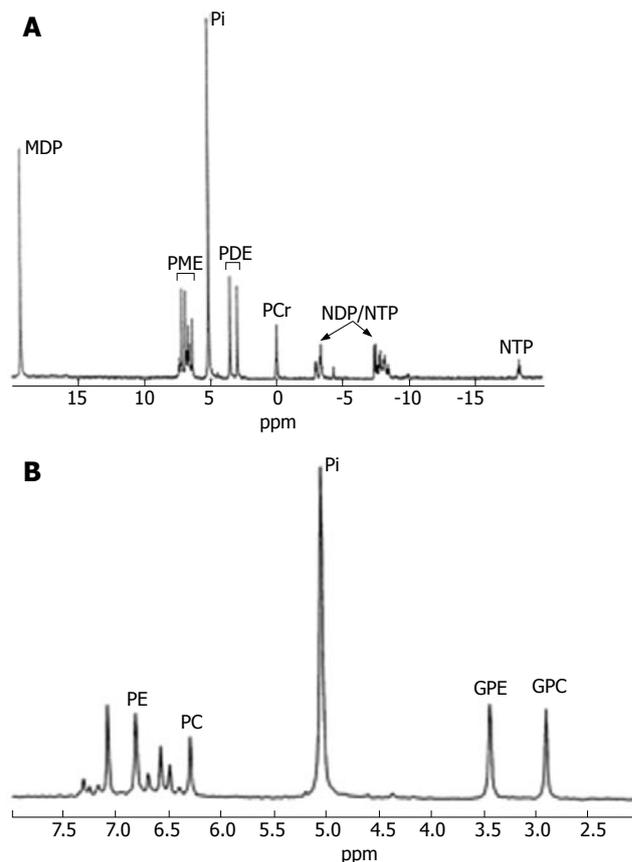


Figure 3 Typical proton decoupled *in vitro* ³¹P MR spectrum of perchloric acid extracted normal liver tissue. **A:** Full spectrum; **B:** PME and PDE regions. PME: phosphomonoesters; PDE: phosphodiester; NTP: nucleotide triphosphates; NDP: nucleotide diphosphate; PE: phosphoethanolamine; PC: phosphocholine; GPE: glycerophosphorylethanolamine; GPC: glycerophosphorylcholine; PCr: phosphocreatine; MDP: methylene diphosphonate. Reproduced from Taylor-Robinson *et al* Gut 1998; 42: 735-743, with permission from the BMJ Publishing Group.

modifications in rates of cell membrane synthesis, breakdown, cell death and regeneration, associated with increasingly rapid cell growth, turnover and development^[13]. Thus, these important phosphorus-containing molecules are intricately involved in the cellular processes linked to cellular destruction, turnover and malignant transformation.

MRS also provides important insights into dynamic metabolic changes in the diseased liver. However, unlike most liver function tests, the information obtained is not dependent on blood flow. Furthermore, most standard liver function tests also depend on measurements of plasma components, rather than assessing markers at the site of disease. Recent studies on hepatitis C suggest that the MRS technique may be useful in monitoring response to treatment with interferon and ribavirin^[8].

Methodology for *in vitro* MR spectroscopy

Sample preparation of body fluids requires routine clinical documentation to ensure that the relevant patient details are adequately controlled or accounted for, including time of sample collection and drug use history. It may be necessary to dilute or buffer the sample, or alternatively to document and account for any spectral changes relating to

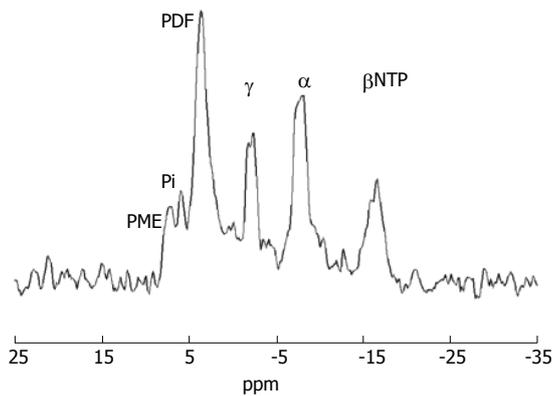


Figure 4 Typical ^{31}P magnetic resonance spectrum from the liver of a healthy volunteer (TR 10000 ms). PME: phosphomonoester; Pi: inorganic phosphate; PDE: phosphodiester; NTP: nucleoside triphosphate; ppm: parts per million. Reproduced from Mullenbach *et al* Gut 2004; 54: 829-834, with permission from the BMJ Publishing Group.

pH or concentration.

Sample storage should not cause a change in metabolite profile. Therefore storage temperature and conditions should be considered, and whether a change in sample temperature alters metabolite profile, for example during a freeze-thaw process^[14,15].

Immediately prior to data collection, a known amount of a NMR standard can be added for NMR referencing and quantification. For example, sodium trimethylsilyl- $[\text{}^2\text{H}_4]$ propionate (TSP) is routinely used as a reference compound for ^1H MRS application. If the sample is required to be uncontaminated by any NMR reference compound for any further analysis, then either a reference compound can be placed in a capillary tube within the sample or an external standard is used.

The temperature of NMR data acquisition is under spectrometer control, so the sample temperature should be specified and constant throughout the study protocol. Many studies are performed at 25°C , but it may be necessary to study intact cells at lower temperatures to minimize the effects of ongoing cellular changes.

MR data acquisition includes adjusting the magnetic field homogeneity within the sample (a process known as 'shimming'). Data averaging is generally required, so as to improve the signal-to-noise ratio in the spectrum. Data can be acquired using pulse-collection methods or one- or two-dimensional NMR data collection protocols^[16]. The water signal is generally suppressed using saturation techniques and/or spin-echo methods, in order to more readily observe the metabolite signals^[17]. Intact tissue specimens may be analyzed using conventional solution MR spectroscopy techniques, by simply placing the biopsy sample within a NMR tube or by chemically extracting aqueous or lipid soluble fractions of the tissues. Studying intact tissue specimens directly within a conventional NMR tube has the disadvantage that magnetic field inhomogeneities within the sample do limit the achievable spectral resolution, but has the advantage that sample preparation is minimal and the sample remains intact and available for further analysis by other complementary techniques. Tissue extract studies have the advantage that

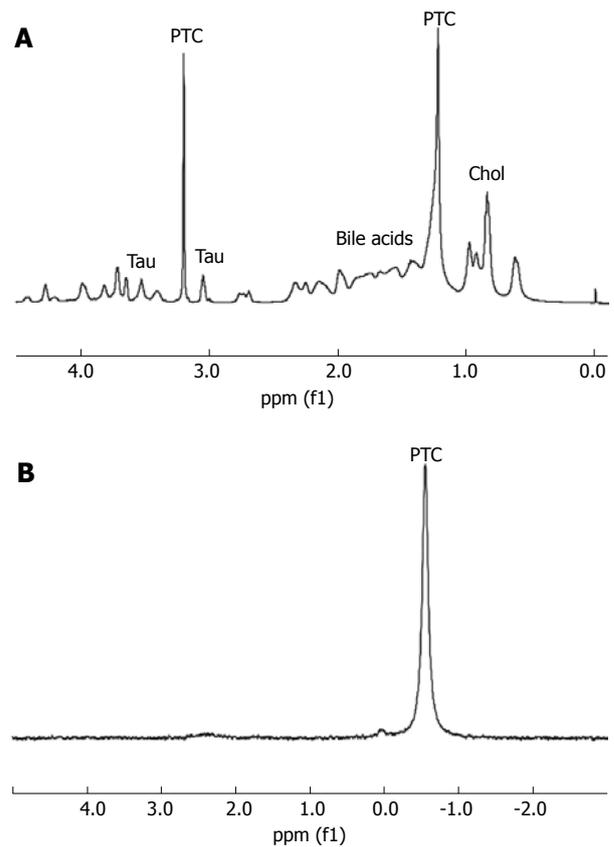


Figure 5 A: ^1H nuclear magnetic resonance spectrum of bile collected at laparoscopic cholecystectomy, after a history of recurrent cholecystitis. The spectrum has no contamination from contrast agents. The major peaks are assigned to phosphatidylcholine (PTC), bile acids, cholesterol (Chol), taurine (Tau) and the reference, sodium trimethylsilyl- $[\text{}^2\text{H}_4]$ propionate (TSP); B: Corresponding ^{31}P nuclear magnetic resonance spectrum. The major peak is assigned to PTC. ppm, Parts per million. Reproduced from Khan SA, Cox IJ, Thillainayagam AV, Bansi DS, Thomas HC, Taylor-Robinson SD. Proton and phosphorus-31 nuclear magnetic resonance spectroscopy of human bile in hepatopancreaticobiliary cancer. *Eur J Gastroenterol Hepatol* 2005; 17: 733-738, with permission from Lippincott Williams & Wilkins, Inc.

the sample which is finally available for MRS study that has taken place after the extraction process, has become homogeneous so that the spectral peaks are well defined. However, the obvious disadvantages include the total destruction of the sample, the additional influence of metabolite solubilities in the extract medium and the relatively large amount of tissue required (upwards of 100 mg). The recently introduced method of magic angle spinning (MAS) MRS can overcome the limitations of both of the above techniques^[18].

Magic angle spinning (MAS) MRS

The line-broadening effects in an intact semi-solid are caused by constraints on molecular motion from cellular architecture, chemical shift anisotropy and dipolar couplings, and these can be reduced by spinning the sample at an angle of 54.7° to the main magnetic field, known as the 'magic angle'. While such MAS MRS techniques have been used for many years in the NMR spectroscopy study of true solids, MAS MRS has only recently been applied to the study of intact tissue^[18]. A distinct advantage is that a MAS MR spectrum can be obtained from as little as 2

mg tissue and the tissue remains sufficiently intact to allow subsequent histological or genetic analysis^[19].

As in conventional MRS studies, accurate determination of metabolite concentrations is pivotal to the success of tissue MAS MRS studies. A number of factors need to be considered for metabolite quantitation in MAS MRS, including possible leakage of metabolites during washing of biopsies prior to analysis^[15], temperature at which the spectrum is acquired and appropriate choice of reference standard. The question of how to define a standard for quantitation is particularly important. For example the tissue water signal measured from a proton spectrum without water presaturation, may be used as an internal standard. Silicone rubber, for example, provides a useful external standard. Alternatively peak area ratios can be used to compare different spectra, but this has limitations as with *in vivo* MRS studies. For example, not only can changes in absolute concentrations be missed by the use of metabolite ratios, but an increase in the area of one peak can have the same result on the area ratio as a decrease in the other. Therefore, relying on the ratio of signal areas may mask metabolite differences inherent in the spectra.

APPLICATIONS OF *IN VITRO* MR SPECTROSCOPY IN LIVER DISEASE

Cellular bioenergetics

Owing to the ubiquity of phosphorus-containing moieties in energy metabolism, ³¹P MRS has been used to assess energy states in living systems^[20]. However, the rapid degradation of phosphorylated nucleotide intermediates *ex vivo*, such as adenosine triphosphate (ATP) and adenosine diphosphate (ADP), has made snap-shot *in vitro* assessment difficult, although efforts have been made to minimize the ischemic time. Oxidation of these metabolites can occur at harvesting, snap freezing, thawing and during tissue extraction. In order to obtain metabolically useful information, the samples should be snap-frozen as soon as possible. In order to eliminate the need for the extraction of aqueous and lipid fractions, MAS MRS techniques could be used but data collection may need to be done at 4°C to minimise further metabolic reactions during data collection.

An alternative methodology to assess bioenergetics is to use an *in vitro* perfusion system. Applications have included the effects of normoxia and anoxia in rat livers^[21], the response to cyanide intoxication^[22] and the response of damaged liver to fructose loading^[23].

Tumours

³¹P and ¹H MR spectra from aqueous soluble metabolites in extract of liver tumours of different types (hepatocellular carcinoma, colorectal metastases and secondary lung carcinoma) have been compared with samples from normal liver tissue and from histologically normal liver of the tumour host^[24]. A significant increase in phosphoethanolamine (PER), phosphocholine (PC), taurine and lactate has been reported, with a reduction in GPE glycerophosphorylethanolamine (GPE) and glycerophosphorylcholine (GPC) in tumours, compared to normal liver controls^[24]. In addition, the spectral changes identified by ³¹P MRS in

tumour tissue have been seen at a lesser magnitude in the tumour host tissues^[24].

Hepatic ³¹P MRS has been translated to the *in vivo* setting by Cox and colleagues and others^[25]. The observed hepatic *in vivo* MR spectrum demonstrated a significantly raised PME/PDE ratio in tumour compared to controls. These peaks are thought to comprise increased PE and PC, and decreased GPE and GPC respectively, as elicited by *in vitro* analysis of tissue extract^[24].

In vitro ¹H MRS in conjunction with a statistical classification strategy has been used by Soper and colleagues to differentiate histologically normal liver, cirrhotic nodules and hepatocellular carcinoma^[26]. Multivariate and pattern recognition techniques enable all data points to be incorporated into the analysis, no matter whether the metabolites comprising the spectral peaks have been identified. In this study, cirrhotic nodules were distinguished from hepatocellular carcinoma in 98% of cases^[26].

Chronic liver disease

Initial hepatic MRS studies of chronic liver disease focused on cirrhosis of varying aetiologies. Human livers with histologically proven cirrhosis have been assessed using *in vitro* ³¹P NMR spectroscopy^[7]. Spectra from these patients with end-stage liver disease, all of whom had tissue obtained at the time of liver transplantation, showed significant elevations in phosphoethanolamine (PE) and phosphocholine (PC) and significant reductions in glycerophosphorylethanolamine (GPE) and glycerophosphorylcholine (GPC), when spectra were compared with those from histologically normal livers. Whether the patient had compensated or decompensated disease did not significantly alter the spectra obtained. Further work^[27] by the same authors correlated such *in vitro* findings with ³¹P MRS *in vivo* and these studies suggest a potential clinical utility for *in vivo* MRS as an addendum to a standard MRI protocol in staging of the end-stage liver disease in patients that are being imaged for surveillance of hepatocellular carcinomas.

More recently, *in vivo* hepatic ³¹P MRS has been used to stratify inflammation and fibrosis caused by hepatitis C virus, compared to histological staging from standard liver biopsy^[8]. The PME/PDE ratio was found to be elevated in mild, moderate/severe fibrosis and cirrhosis compared to normal, healthy volunteers. These changes have been thought to represent increased cell membrane turnover, although differences between means for each group were statistically significant, there was some overlap between the patient groups^[8]. While histology remains the gold-standard, sampling error is inherent in the technique and it has been postulated that hepatic MRS could potentially provide a more accurate representation of the disease process, owing to the fact that it provides metabolite information from most of the liver^[8]. Further studies directly correlating *in vivo* and *in vitro* MRS findings are awaited in this context.

While *in vivo* hepatic ³¹P MRS has been shown to correlate closely with disease severity in hepatitis C, its availability as a technique is not widespread. *In vivo* hepatic ¹H MR spectroscopy would be more generally applicable on standard MRI scanners. Cho and colleagues have used this

technique to stratify chronic hepatitis compared to histology^[28]. They were able to detect differences between groups, but the signal-to-noise ratio was low and assignment of the spectral peaks is open to debate^[29]. A rational approach would be to identify the change in spectral peaks *in vitro*, using ¹H MAS MRS, prior to translating the technique to the *in vivo* setting. With this in mind, Martínez-Granados and colleagues performed ¹H MAS MRS on needle biopsies of liver tissue from 16 patients with chronic hepatitis or cirrhosis and one specimen from autopsy, nominated a “normal” control. High quality spectra were obtained and a number of metabolites assigned to previously unidentified peaks. Data collection was carried out at 4°C to minimize tissue degradation. Differences in signal intensities between disease states and “normal” liver were noted, particularly increases in mobile fatty acids and glycogen in the former. However, the “normal” liver was harvested 24 h post mortem, so assumptions of metabolic normality are questionable^[30].

Hepatic steatosis

The intrahepatocellular lipid (IHCL) component of lipid extracts from steatotic liver specimens as assessed by ¹H MR spectroscopy has been calibrated with *in vivo* MR spectroscopy measurements to estimate the lipid volume fraction in fatty liver disease^[31]. There is an agreement with CT estimations but histomorphometry appears to underestimate the fat volume in samples. Szczepaniak and colleagues used localised *in vivo* ¹H MRS in 2349 patients to assess the hepatic triglyceride content and estimated the prevalence of hepatic steatosis in the study group as 33.6%^[32]. Further MRI studies *in vivo* have demonstrated a close relation between hepatic steatosis and body adiposity, and a close correlation between MRI estimation of adiposity and histological assessment in two of these patients^[11]. Although studies have shown a close association between *in vivo* estimates and biopsies, *in vitro* MRS assessment of lipid content in liver biopsies by MAS MRS would allow direct comparison with histology, reducing the effect of sampling error^[33].

Orthotopic liver transplantation

Liver transplantation is used as a definitive therapy for acute hepatic failure, severe chronic liver disease and some cases of hepatocellular carcinoma. Demand for donor livers is high and suboptimal specimens may be used. At present there is no reliable non-invasive method to assess the viability of livers between organ harvesting and implantation. A program of research to address these problems demonstrates translational techniques from animal models *in vitro* to the clinical environment.

In vitro and *ex vivo* MRS studies of animals have established a model, which follows human organ harvesting and storage protocols^[34,35]. Rapid reductions in ATP levels, readily measured by *ex vivo* ³¹P MRS have been seen in a pig model compared to rodent models and levels could be replenished by hypothermic reperfusion^[34]. Now that MR probes have been designed to fit within organ retrieval boxes, clinical studies of organs within a transplant program are required^[36].

In a preliminary study, Duarte and colleagues used *in vitro* ¹H MAS MRS to assess biopsies taken at three time-points from six livers, before removal from donors, during cold perfusion and following implantation into the recipient^[33]. The biopsies with the highest concentration of peaks reflecting fatty acyl chain (triglyceride) resonances were also identified as those also estimated to have the highest fat content on histological analysis. Other metabolites were identified, including glycerophosphocholine (GPC), which were reported to decrease from pre- to post-transplant^[33]. However, in further studies such spectral changes need to be correlated with a range of clinical endpoints, including the pre-morbid clinical history of the liver donor, the pre-transplantation clinical history and nutritional status of the recipient, the subsequent post-transplantation liver function tests, pre-and post-transplantation indices of nutrition in the recipient and ultimately, the final clinical outcome.

Moving towards using MRS as a non-invasive methods for assessing graft dysfunction following transplantation, Taylor-Robinson and colleagues used correlative *in vitro* MRS of liver biopsy material and *in vivo* whole-body clinical hepatic ³¹P MRS to examine chronic ductopenic rejection of human liver allografts and noted an increased PME/NTP metabolite ratio reflecting associated altered phospholipid metabolism^[37]. The study also included electron microscopy of liver tissue and the alteration in the phospholipid component was judged to be related to a change in biliary phospholipid excretion in these cholestatic patients^[37]. Such an increase in the *in vivo* PDE resonance, seen in the *in vivo* hepatic MR spectrum is not a specific finding in patients with chronic allograft rejection, because a similar, albeit less marked change has been found in patients with primary biliary cirrhosis and obstetric cholestasis^[38,39].

Iron studies

Iron overload may be a result of iatrogenic causes such as multiple blood transfusions in beta-thalassaemia, or of metabolic causes such as hereditary haemochromatosis, and is commonly associated with liver dysfunction. At the current time, hepatic iron stores are still usually estimated using liver biopsy with the associated risks that this procedure carries, but are readily studied by MR, since paramagnetic iron compounds cause magnetic inhomogeneities, which shorten the nuclear relaxation time^[40,41]. However, imaging is difficult on account of rapid relaxation caused by these iron moieties. In addition, use of different MR sequences is required and equipment may require recalibration. Relaxometry allows measurement of relaxation times and provides information as to iron-proton interactions. An *in vitro* approach allows direct quantification of iron following MR analysis^[42]. *In vivo*, Wang and colleagues demonstrated single voxel MRS measurement of T₂ in liver iron overload that correlates strongly with iron quantification from biopsy and overcomes the difficulty of lack of detectable signals in conventional MRI^[43]. Furthermore, Gandon and colleagues have proposed the use of a liver to muscle intensity ratio, which is transferable between equipment and sequences^[44].

Gene therapy

Gene therapy offers the opportunity to replace defective genes in phenotypically abnormal tissue with recombinant genes. Integration into the host cell genome allows expression of the desired protein. However, methods of conveying the gene to the required location and monitoring delivery and expression are required. Viral vectors and non-viral means such as naked DNA, liposomes and molecular conjugates have all been used. Expression varies with time and between tissues. MR techniques using MR spectroscopy and/or MR imaging, offer non-invasive methods of monitoring expression^[45,46]. As in drug monitoring techniques where a MR detectable moiety or metabolite is linked to the active drug, genes expressing markers may be combined with the therapeutic gene. Phosphoarginine produced by the enzyme arginine kinase in *Drosophila*, but not present in mammalian skeletal muscle, is expressed in mouse skeletal muscle and detected by ³¹P MRS following injection of an adenovirus vector^[47]. The expression in neonatal mice could continue for up to eight months. This demonstrates elegantly the principle of 'marker metabolite' using xenogenetic material. This principle has been demonstrated *in vitro* using a hepatocyte cell line^[48]. Hepatocytes do not express creatinine kinase so phosphocreatine has not been seen on the hepatic MR spectrum. Integration of the cytoplasmic creatine kinase into hepatocytes *in vitro* could lead to detection of phosphocreatine by ³¹P MRS, raising the possibility that combined with a hepatotropic gene delivery system, gene expression may be monitored *in vivo* through a MR-visible marker.

APPLICATIONS OF *IN VITRO* NMR SPECTROSCOPY IN BILIARY DISEASE

Introduction

Bile is predominantly an aqueous solution containing numerous constituents. Bile acids (BA), phosphatidylcholine (PTC) and cholesterol are the predominant lipid components of bile. Other components include electrolytes, organic anions (bilirubin), plasma proteins, hepatocyte proteins, peptides and amino acids, nucleotides, heavy metals and vitamins, xenobiotics and toxins^[49]. In health the concentration of these biliary constituents is tightly controlled.

Primary bile acids such as chenodeoxycholic acid (CDCA) and cholic acid (CA), and secondary bile acids such as lithocholic acid (LCA) and deoxycholic acid (DOCA) are conjugated with the amino acids taurine and glycine and secreted as sodium or potassium salts into the biliary canaliculi via the ABC biliary transporter proteins^[50]. Phosphatidylcholine, the predominant biliary phospholipid, is synthesized in hepatocytes and transported into the biliary canaliculi by the flippase multidrug resistant protein 3 (MDR3)^[51]. Its main function is to form mixed micelles with primary and secondary bile acids and cholesterol essential for the emulsification of fats. More recently it has been shown to be cytoprotective to the biliary epithelium^[52]. Cholesterol is solubilized by the formation of vesicles with PTC or mixed micelles with bile salts and PTC.

Sampling of bile for diagnostic purposes has become a common clinical practice since the introduction of endoscopic retrograde cholangiopancreatography (ERCP). Bile research has advanced in the last decade mainly as a consequence of the alarming rise in incidence of biliary tract cancer (cholangiocarcinoma)^[53-55].

Advanced cytological techniques such as digital image analysis and fluorescence *in situ* hybridization (FISH) of bile are being used to improve the diagnostic accuracy of cholangiocarcinoma in certain experimental units^[56]. More recently proteomic analysis of cholangiocarcinoma bile has identified 87 unique proteins including several novel proteins whose functions are unknown and a large number of proteins not previously described in bile^[57]. Advances in molecular research and imaging technologies have vastly improved the diagnostic sensitivity and specificity in this area but there is still a clear need to identify novel, highly sensitive and specific biomarkers for fluid-based detection of biliary tract cancer, as well as other diseases of the biliary tree. Metabolic profiling of bile using *in vitro* MRS is a valuable experimental tool for the identification of such early biomarkers of the disease.

Bile physiology by MRS

The majority of MRS studies on bile have been carried out using ¹H MR spectroscopy. The hydrophobic association of conjugated bile acids and biliary lipids in micelles has been confirmed in early MR studies^[58]. Bile acids have also been quantified in both animal and human bile using *in vitro* ¹H MRS. Rat bile studies with ¹H MRS have derived peak assignments for C-18 methyl proton of bile acids at 0.7 ppm and the C-19 at 0.9 ppm, while the taurine moiety of taurine-conjugated bile acids resonated at 3.1 ppm and 3.5 ppm, respectively^[59]. Conjugated bile acids in human gallbladder bile have been quantified using two dimensional MR^[60]. Amide (-NH) proton resonances in glycine- and taurine-conjugated bile salts are in the region of 7.8-8.1 ppm^[60].

More recently, ¹H MRS has demonstrated cholesterol in human bile that can be differentiated from other lipid components in bile^[61]. It has also been utilized for studying the effects of cholesterol on the fluidity of human gallbladder bile as well as for quantifying micellar PTC concentrations^[61,62].

³¹P NMR spectroscopy has been used to quantify phospholipids in red cell membranes and in model bile salts^[63]. Such ³¹P MRS studies have shown higher concentrations of PTC and Pi in gallbladder bile compared to canalicular bile^[64].

MR spectroscopy of bile in disease

The biliary epithelium is exposed to numerous constituents of bile. Bile acids, PTC and cholesterol are of particular importance in disease if the cytoprotective mechanism of cholangiocytes is disrupted^[65]. Biliary disease has a major effect on biliary composition as the level of cellular function determines the production and biochemical constituents of bile. Biliary composition of these lipids also varies in cholestatic diseases of the liver and malignancy of the biliary tree. Current applications of

MRS in biliary disease include assessment of bile content in cholesterol gallstones, rejection in liver transplantation, primary biliary cirrhosis and biliary tract malignancy, as well in biliary excretion of xenobiotics^[64,66-69].

MRS has been used to assess the distribution of biliary lipids between vesicles and micelles, which is believed to have a role in gallstone disease, and there is evidence that cholesterol from vesicular aggregates may be responsible for the deposition of cholesterol stones in the gallbladder^[70]. Changes in the pattern of fatty acids of PTC with an increase of arachidonic acid have also been observed in bile from patients with gallstones^[71]. Fusion and aggregation of phospholipid-cholesterol vesicles which form liquid crystalline droplets leading to nucleation of cholesterol monohydrate crystals are thought to be responsible for the formation of cholesterol gallstones^[70]. A selective reduction in biliary phospholipids has been suggested to be responsible for cholesterol gallstones in certain populations^[72].

Initial ³¹P MRS studies on bile from patients with primary biliary cirrhosis have shown reduced levels of PTC and Pi when compared to bile from healthy volunteers^[64], but the significance of these findings still remains to be determined in more extensive studies.

In vitro MRS has also been demonstrated to effectively identify and quantify xenobiotic metabolites in human and animal bile. Dioxins have been identified in biliary-cannulated rodents^[67].

In vitro ¹H MRS analysis of bile has also been applied in human liver transplantation in an attempt to assess donor liver integrity. Melendez and colleagues studied twenty-four hepatic bile samples from eight liver donors^[69]. The livers from two donors were steatotic on histological analysis, while the rest were normal. ¹H MRS analysis could show more intense PTC resonances in bile from steatotic donor livers, compared to bile from histologically normal donor livers. Seventeen hepatic bile samples from four recipients collected immediately after donor liver reperfusion were also analyzed by ¹H MRS and showed bile from donor livers with good early graft function had a progressive increase in the bile acid peaks which represents restoration of bile flow. When compared to grafts with early graft dysfunction, the relatively reduced bile acid peaks in these spectra suggested slow recovery of bile secretion. In this study a total quantification of bile acids was not feasible due overlapping signals from other biliary lipids, notably cholesterol and PTC^[69].

Our preliminary studies have revealed differences in phospholipid metabolites that may help distinguish between malignant and non-malignant causes of pancreaticobiliary obstruction. A reduced PTC resonance was seen in the bile from the majority of patients with hepatobiliary cancer compared to bile from patients with non-malignant indications for ERCP. This preliminary observation was confirmed by significant differences in the peak area ratios of PTC, referenced to the TSP standard in the ¹H MR spectra ($P = 0.007$)^[68].

Nishijima and colleagues have observed a lactate peak in bile spectra from patients with hepatic and biliary malignancy but not in bile from patients with non-malignant disease or bile from healthy controls^[73]. The

significance of this finding has yet to be determined in larger studies, where the collection and storage of bile for analysis are performed according to uniform protocols without potential contamination from ERCP contrast agents or the uncertainty of a long storage time that may lead to lactate accumulation.

FUTURE STUDIES

Although MRS is primarily a research tool and its use to study hepatobiliary disease is a relatively new area, the ability of both *in vivo* and *in vitro* MRS to provide quick, repetitive, and non-invasive assessments of organ function raises several possible future development areas, including non-invasive diagnosis and staging of disease.

In vitro MRS holds a particular promise in the metabolic profiling of body fluids, such as urine, plasma and bile to pinpoint the potential disease mechanisms and to assess the response of the body to treatment regimens. This is of particular relevance when patients are having *in vivo* MRS studies, since an *in vivo/in vitro* correlation of the metabolite profile obtained may highlight disease processes and inform further genetic profiling studies^[39].

Several clinical areas may be of potential interest. In the oncology field, phospholipid profiling to assess changes by *in vivo* ³¹P MRS may be useful in monitoring responses to the treatment of hepatic tumors, with co-temporaneous *in vitro* MRS analysis of plasma and urine. For example, chemoembolization of hepatic tumors has been found to correlate with a decrease in PME and increase in ATP concentrations using *in vivo* hepatic MRS^[74].

Correlative *in vitro* MRS of body fluids and *in vivo* hepatic MRS techniques can also be applied to chronic liver disease to monitor liver fibrosis and progression towards cirrhosis. With increasing prevalence of alcohol-related liver disease, obesity-related liver disease (non-alcoholic hepatitis or NASH) and viral hepatitis, such as hepatitis C in particular, a reliable and repeatable monitoring system is required that obviates the morbidity and mortality associated with liver biopsy. *In vivo* and *in vitro* MRS may hold some of the answers to this, but further, larger scale studies are required to assess the true utility of these techniques with respect to other methodologies, such as microbubble contrast-enhanced ultrasound, ultrasound elastography and use of serological markers of fibrosis^[75,76].

Future technical advances will boost the clinical potential of *in vivo* MRS, for example improving the delineation of multi-component signals, such as PME and PDE, with proton decoupling^[77]. Increasing magnetic field strengths for *in vivo* studies may also provide better signal-to-noise and increased resolution, and 3T magnets are becoming steadily more available. Furthermore, *in vivo* ¹H MRS may find a role in monitoring hepatic lipid content in interventional treatment studies of patients with non-alcoholic fatty liver disease in the future. *In vivo* ¹³C MRS for measurement of lipid metabolism, glycogen storage and gluconeogenesis looks promising for the future, but current sensitivity issues mean that this capability is currently confined only to a few centers^[78-80]. *In vitro* MRS will find a correlative role in this context for screening plasma and urinary metabolites.

With respect to *in vitro* applications in bile, the ratio of taurine to glycine conjugates of bile acids and conjugated to unconjugated bile acids varies in hepatobiliary disease, and it has been widely accepted that elevated levels of bile acids in hepatocytes are toxic, leading to cholestasis^[65]. More recently, the role of bile acids in cholangiocytes has been highlighted. *In vitro* studies on bile acids has demonstrated that they can act as ligands for the epidermal growth factor receptor on cholangiocytes and *via* the mitogen-activated protein kinase cell signalling pathway, leading to disordered cell cycling and cholangiocyte proliferation^[81]. The measurement of bile acids is therefore necessary for an understanding of the pathophysiology of these diseases. *In vitro* MRS may find a useful role in screening bile in this context.

It is true that the significance of *in vivo* MRS needs to be assessed in larger studies with greater numbers of patients with various hepatobiliary diseases. Correlative *in vitro* NMR spectroscopy will help to elucidate some of the conundra. Trials in large populations in well-defined clinical settings are needed to determine if both *in vivo* and *in vitro* MRS can provide independent diagnostic and prognostic indices in management.

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EDITORIAL

Contribution of genetics to a new vision in the understanding of inflammatory bowel disease

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Abstract

Inflammatory bowel diseases (IBD), such as Crohn's disease (CD) and ulcerative colitis (UC), are chronic inflammatory autoimmune conditions of the gastrointestinal tract. Other organs, such as the eyes, skin and articulations, are often affected and IBD may be accompanied by other diseases of autoimmune origin. There is no single etiological factor responsible for the onset of IBD. Recent advances in genetics and in the molecular mechanisms of the proteins coded by these genes have given rise to a new vision in understanding these complex diseases. Activation of specific genes that affect antigen presentation and the handling of cells by innate immunity may lead to autoimmunity with the consequent activation of the major histocompatibility complex (MHC) and multiple cytokines involved in the regulation of acquired immunity. In this review IBD is described as a constellation of diseases that can best be classified as barrier diseases. This vision, developed by Kiel in Germany, includes the idea that changes in our environment due to the westernization of civilization have not been met with adaptation of the innate immune system, and this has given rise to autoimmune diseases. These diseases affect 1-5 of 1000 individuals and represent a major burden on the national health systems of many countries on different continents. On a world scale, a major challenge is to generate interventions to prevent the development of these diseases in Asia, Latin America and Africa.

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Key words: Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Genetics; Autoimmunity; Major histocompatibility complex

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INTRODUCTION

In less than five years after the discovery of the first gene involved in susceptibility to Crohn's disease (CD), the initial concept that CD is a multifactorial and polygenic disease has been consolidated^[1,2].

Although the detailed functions of the *NOD2* or *CARD15* gene of chromosome 16 is not entirely clear, their relationship to intestinal flora, Toll receptors (TLR), and other intra-cytoplasmatic receptors of the NOD family together with their relation with NF- κ B, has made clear that the innate immune response is of paramount importance in the pathogenesis of CD^[3-5].

Two years ago it was suggested that the relationship between *TLR-2* and *NOD2* genes could explain the balance between activation of superficial receptors of the epithelial and dendritic cells to stimulate the production of NF- κ B and *NOD2* as inhibitors in order to prevent chronic disease^[6-8]. According to this concept, individuals with mutations in the *CARD15* gene are not able to control the intestinal inflammation and this induces a TH1 immune response^[9]. Recently, however, mutations in the terminal N of the gene in a region rich with leucine repeats have been transfected into the *NOD2* region of mice^[10]. Surprisingly, when these mice are challenged with muramyl dipeptide (MDP) they generate considerable production of NF- κ B and IL-1 β ^[10]. Also the transfection of these mutations in HEK293 cells results in increased transcription of TNF- α , which suggests that different genes contribute to susceptibility to the disease and differences in manifestations of CD^[11].

The fact that *NOD2* is preferentially expressed in Paneth cells in the ileocaecal region probably explains the strong association between these mutations and this disease localization^[12,13]. This region is rich in defensins, which are natural antibiotics that contribute to the mucosal barrier and innate immunity^[14]. It is also now known that patients with mutations in the *CARD15* gene also have less α -defensins. This defect probably contributes to the role of intestinal flora in inducing and/or maintaining inflammation^[15]. Preliminary results of a study carried out by the Stange group in Germany suggest that defensin deficiency is genetically determined. The defensin family is more complex and richer than originally described and its distribution varies within the gastrointestinal tract. The β -defensins are localized in the colon and defective β -defensins could contribute to the colonic localization of IBD^[16]. It is also possible that different defensins may be specific to CD and UC variations^[17].

These observations demonstrate the fine-tuning of

molecular biological responses of the gastrointestinal tract and the complexity of interactions among different genes on different chromosomes. Another example of complexity is gene polymorphisms of the gene inhibitor of plasminogen (PAI-1) in combination with *CARD15* mutations have an influence in the development of CD. Those patients who have mutations of *CARD15* and carry the *PAI-14G/4G* genotype develop a stricturing phenotype (OR, 4.64; 95% CI, 1.26-17.05)^[18].

Despite the well-demonstrated and replicated role of *CARD15* mutations in the susceptibility for CD in the majority of Caucasian populations^[19-23], no mutations have been found in Asian^[24-27] and in several European populations. In Scotland, Ireland, Galicia, Sweden and Finland the carriership of mutations in the *CARD15* gene is less frequent^[28-32]. An interesting phenomenon was recently reported in monozygotic twins. In Sweden, the number of carriers of these mutations was as low as in the general population^[32]. However, in Denmark 40% of the monozygotic twins carried *CARD15* mutations, which was a higher rate than in the Danish population with CD^[33]. This corroborates the observations in Finland where the 1007fs allele frequency was higher in familial CD than in non-familial cases with CD (10.9% vs 3.5%; $P < 0.01$)^[30]. These observations underscore the incidence of genetic variability and the importance of studying healthy controls in the general population.

RELEVANCE OF OTHER GENES

Several other genes on other chromosomes are involved in determining susceptibility to CD. Two interesting genes, one on chromosome 5 and one on chromosome 10, contribute to the new vision of the genetics of CD. On chromosome 5, the *SLC22A4/SLC22A5* haplotype codes for molecules involved in cationic transport, other solutes and carnitine (OCT-1 y OCT2)^[34-36]. In some populations, an epistatic interaction has been found to exist between the *CARD15* mutations and the 250 KB region of 5q31^[37,38]. The other interesting gene in this context is the *DLG5* on chromosome 10, which is a gene that is important in the scaffolding of the epithelial cell^[39,40].

The 250 KB region of 5q31 and the HLA region of 6p21 contain several genes that are of paramount importance in the regulation of the immune response and probably contribute to the phenotype of the patient with IBD. The *HLA-DRB1*0103* allele is associated with UC and with the colonic localization of CD^[41,42].

Carriers of *HLA-DRB1*0103*, *B*35* and *B*27* have higher risk for arthralgias/arthritis in some of the greater articulations and *HLA-B*44* carriers are at higher risk for symmetric poly-arthritis^[43,44].

A meta-analysis of 1068 CD patients has also implicated the genes described above in the development of CD and this study identified other regions of potential relevance on chromosomes 2q, 3q, 17q and 19q^[45].

THE CONTRIBUTION OF GENETICS TO A NEW VISION

In the new vision of CD, several genes are involved in the

maintenance of the intestinal barrier, such as scaffolding genes (*DLG5*^[46]), genes involved in the transport of key molecules for the homeostasis or exclusion of toxins (*SLC*^[47] and *MDR1*^[48,49]), genes involving the sensing of bacteria, both on the surface (TLR, CD14) and intracytoplasmatically (*CARD15*, *CARD4* and *CARD8*^[50-52]). Whether a specific gene for regulating permeability exists, as has been suggested on chromosome 19, remains to be demonstrated. The combination of gene rich regions of chromosome 5 and 6 involved in regulating the immune response may contribute to the phenotype of the disease.

In summary, in less than a decade the genetics of IBD has evolved from epidemiology to molecular biology, and from observational studies to functional studies. The challenge for the coming years is the discovery of gene-gene interactions and gene-environment interactions.

A delineation of phenotypes based on genetic and molecular mechanisms will improve diagnoses and more accurate prognoses. This knowledge, together with advances in the understanding of the phenomenon of tolerance and the disruption of this mechanism in the understanding of chronic inflammation of the gastrointestinal tract, will lead to the design of better and novel therapeutic strategies. This should be the basis for effective drug development in addition to increasing knowledge generated by pharmacogenetics and pharmacogenomics.

Regarding the role of the environment in those individuals with a genetic susceptibility, new findings on epigenetic effects from long-term follow-up in monozygotic twins will open a new area of investigation. Investigators at the Spanish National Cancer Centre (CNIO) in Madrid have shown that older monozygotic twins exhibited differences in their overall content and genomic distribution of 5-methylcytosine DNA and histone acetylation. These epigenetic changes affect their gene-expression patterns and may explain the well-known disease discordance in these cases of monozygotic twins^[53].

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REVIEW

Host susceptibility to persistent hepatitis B virus infection

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Abstract

Genetic epidemiology researches such as twin studies, family-clustering of hepatitis B virus (HBV) infection studies and ethnic difference studies have provided the evidence that host genetic factors play an important role in determining the outcome of HBV infection. The opening questions include which human genes are important in infection and how to find them. Though a number of studies have sought genetic associations between HBV infection/persistence and gene polymorphisms, the candidate gene-based approach is clearly inadequate to fully explain the genetic basis of the disease. With the advent of new genetic markers and automated genotyping, genetic mapping can be conducted extremely rapid. This approach has been successful in some infectious diseases. Linkage analysis can find host genes susceptible to HBV and is of great clinical importance.

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Key words: Hepatitis B virus; Susceptibility; Association study; Linkage study

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INTRODUCTION

Hepatitis B virus (HBV) infection is a serious global health problem, with 2 billion people infected worldwide, and 350 million suffering from chronic HBV infection. HBV infection results in 500 000 to 1.2 million deaths per year

caused by chronic hepatitis, cirrhosis, and hepatocellular carcinoma and is the 10th leading cause of death worldwide^[1]. The mechanisms of persistent HBV infection are not fully understood, but they seem to involve several aspects and the genetic component, in particular, is still controversial^[2]. Early studies by Blumberg *et al*^[3] have also suggested a recessive mode of inheritance for HBV viral persistence, but this is perhaps an oversimplification giving the more recent advances in knowledge of the effect of maternal viral infection and the transmissibility of the virus^[4].

Generally, exposure to HBV can cause a broad spectrum of infection^[5]. Ninety to ninety-five percent of adults infected with HBV can eliminate the virus and only 5%-10% of them become chronic HBV carriers, 20%-30% of chronic HBV carriers develop chronic hepatitis B (CHB) and 5% of them develop liver cirrhosis and hepatocellular carcinoma in a long term of disease course. Some rare cases result in a fulminant infection in which the liver is rapidly overwhelmed and ultimately fails. What factors determine why one develops a life-threatening infection, whereas another carries HBV as a harmless commensal or limits the infection to a clinical trivial episode? There is evidence that host genetic factors play an important role in determining the outcome of HBV infection^[6,7].

EPIDEMIOLOGY EVIDENCE FOR HUMAN GENETIC SUSCEPTIBILITY TO PERSISTENT HBV INFECTION: TWINS, FAMILY-CLUSTERING OF HBV INFECTION AND ETHNIC DIFFERENCES

Twin studies

Studies of susceptibility to diseases in identical and non-identical twins are extremely useful in evaluating the importance of inherited *vs* environmental factors in disease susceptibility^[8]. If the concordance rates for infection and clearance of HBV are significantly higher in monozygotic (MZ) than in dizygotic (DZ) twins, the process of HBV infection and persistence is more genetically decisive. Lin *et al*^[9] studied 289 pairs of MZ twins, 102 pairs of DZ twins and 375 pairs of age-sex-matched singleton controls and found that there is a significant difference in the concordance of HBV infection between MZ and DZ twins and controls, suggesting that the genetic influence occurs in response to HBV infection. Xu *et al*^[10] also found that not only the concordance rate of infection, but the concordance of clinical phenotype and serological

patterns between MZ and control groups is significantly different, indicating that genetic factors influence not only susceptibility to infection but also clinical outcome.

Genetic factors not only influence host response to HBV infection, but also affect the response to HB vaccine. Hohler *et al.*^[11] prospectively studied and vaccinated 202 twin pairs with a combined recombinant HBsAg vaccine and found that the heritability of anti-HBs immune response is 0.61, which means that 60% of the phenotypic variance of responsiveness to HB vaccine can be explained by genetic effect and 40% by environmental effect.

Family-clustering of HBV infection

Most of us do not inherit single-gene diseases. We all, however, inherit slightly different variants of each of our pairs of 30 000 genes. These differences may determine whether we are more or less likely to develop particular health problems or diseases than other people. Genes are shared within families. Because we inherit genes from our parents, a parent who has inherited a particular gene mutation generally means that each child has a fifty-fifty chance of having the same mutation. In fact, many cases of diseases not showing the clear inherited patterns of single-gene diseases, show family clustering patterns that are due, at least in part, to genetics. Substantial genetic epidemiology studies indicate that HBV spreads in families. The familial occurrence of HBV infection has been well established in some ethnic groups. Ohbayashi *et al.*^[12] have reported 3 Japanese families in which 36 of the 54 members are HBsAg positive. Of these, some are healthy carriers while others have liver cirrhosis and hepatocellular carcinoma. Similar observations have been reported in American^[13], European^[14] and Asian^[15,16] continents.

This observed familial clustering may stem from inherited defects in specific genes, from shared environmental exposures among family members or from interaction between specific genetic and environmental factors. If a trait has a genetic basis, the relatives of affected individuals will be affected more frequently than the relatives of unaffected people, and the prevalence of disease decreases from monozygotic (MZ) twin to the first-, second- and third-degree relatives. If the disorder has an environmental basis only, the possibility of infection in each family member is equal^[17]. Tong *et al.*^[18] reported that HBV markers are detected more frequently in blood relatives than in non-blood relatives of the index cases in family. Wang *et al.*^[19] also showed that HBsAg carrier rate decreases in the order of the first, second and third degree relatives, indicating that it is the defect gene shared by family members that produces the epidemiological characteristics of family-clustering HBV infection.

Ethnic differences

Another method used to investigate the role of host genetics in infectious diseases is to look for differences in clinical disease and immune response between different ethnic groups having equal exposure to the same pathogen. Carrilho *et al.*^[20] determined the frequency of HBV markers of genetically related (consanguineous) and non-genetically related (non-consanguineous) Brazilian families of Asian

origin and Western origin and found that the occurrence of HBsAg is significantly higher ($P < 0.0001$) in family members of Asian origin (81.8%) than in those of Western origin (36.5%), which is in line with the high HBsAg prevalence in Asian countries and the relatively low HBsAg prevalence in Western countries^[21]. Though the Asians live in Brazil, a country with a low HBsAg prevalence, and the environment has changed, disease-related genes remain shared within the ethnic group, indicating that Asians possess the HBV susceptible gene(s). This is why they are more susceptible to HBV.

Tong *et al.*^[18] tested family members of Asian and non-Asian patients for HBV markers, and found that Asian family members have a significant increase in HBsAg (34% higher) and antibodies to HBsAg or to hepatitis B core antigen (50% higher) compared with the non-Asian family members. Moreover, birthplace, either in Asia or in United States, does not influence the frequency of antigenemia. In China, the prevalence of HBsAg is 19.1% in Mongoloid populations^[22], and 10% in Chinese Han populations in the same area. These studies have provided important insights into the fact that different ethnics in the same region have different HBV epidemiological characteristics and the same races in the different region share the same prevalence of HBV markers, indicating that genetic factors may play a role in maintaining the frequency of HBV infection and persistence. Moreover, molecular epidemiology study has identified several genetically determined differences between races.

Taken together these epidemiological data provide strong evidence for a genetic predisposition to HBV infection and raise the questions of which human genes are important in infection and how to find them.

TWO METHODS USED TO IDENTIFY HBV SUSCEPTIBLE GENES

Analysis of the human genome has focused primarily on variations that occur between people in their DNA sequence^[23]. Because these differences contribute to the differences in our susceptibility to developing specific diseases, naturally occurring genetic variations in the human genome are frequently found (about every 3 to 500 bp) most often in the form of a change from one base to another, namely a single nucleotide polymorphism (SNP)^[24]. Other common forms of variation include microsatellite where a short sequence, usually a dinucleotide repeat is bound, so that one person might have 10 and 12 copies of the repeating motif and others have 9 and 11 copies. If the repeating sequence is longer, the motif is known as a minisatellite^[25]. They are widely used to determine similarities and differences of human and hunter disease-related genes. Because this kind of genetic variations often varies between individuals (i.e., it is highly polymorphic), microsatellites are particularly informative in the genetic sense^[26]. Analysis of genetic susceptibility to HBV infection aims to link these DNA variations (the genotype) with a particular HBV infection (the phenotype). HBV infection and clearance are complex traits^[27], meaning that the genetic contribution to them is not inherited in a

Table 1 Gene polymorphisms associated with clearance of HBV infection

Gene/loci	Population	Sample size	P value	Reference
HLA A 0301	Caucasian	563	0.0005	[30]
HLA -DRB1 1302	Caucasian	563	0.03	[30]
HLA-DRB1 1302	Gambian	638	0.012	[31]
HLA-DRB1 1101/1104	Chinese	190	0.0145	[32]
HLA-DQA1 0301	Chinese	190	0.0167	[32]
HLA-DR13	Korean	1272	< 0.001	[33]
TNF-alpha-238 GG genotype	Chinese	895	0.041	[34]
TNF-alpha-308 A	Korean	1400	< 0.001	[35]
TNF-alpha-857 TT genotype	Chinese	355	0.02	[36]
CTLA-4-1722 C			0.06	[37]
CTLA-4+49 G			0.02	[37]
CCR5 59029 G allele	Chinese	377	0.001	[38]

simple Mendelian manner and several polymorphic genes exert effects on the outcome^[28]. Many possible approaches to mapping the genes underlying complex traits fall broadly into two categories: candidate gene- based association studies and genome-wide linkage studies^[29].

Association studies

Association studies compare the frequency of alleles or genotypes of a particular variant between disease cases and controls to link the genotype with the particular phenotype. Such studies are widely used to investigate inflammatory and infectious diseases. Repeat sequences, such as those of microsatellites, lend themselves less well to association studies because they are intrinsically unstable and may undergo considerable mutations over successive generations and disease-modifying polymorphisms may have arisen many hundreds of generations previously. SNPs, on the other hand, are stable, common and increasingly amenable to high throughput automated genotyping. A number of studies have sought genetic associations between HBV infection/persistence and gene polymorphisms (Tables 1 and 2).

The huge variation in clinical response to identical infecting pathogens is due to the combined effects of genetic variation both in the infecting pathogen and in the infected host^[44]. Its ability to mount an effective immune response to infection is a powerful evolutionary selection pressure, contributing to human genetic diversity. The advantage of a flexible immune response, allowing an efficient response to diverse pathogens without damage to the host, is reflected in marked genetic variability of immune-related genes among (both in DNA sequence and in protein structure) in the entire human genome^[45].

The prototype region for genetic association studies is the human leukocyte antigen (HLA) loci involved in antigen processing and presentation. HLA associated with infections such as AIDS^[46], tuberculosis^[47], leprosy^[48], malaria^[49] and persistence of hepatitis-C virus^[50] has been well-described. This is most obvious within the HLA region, where functional variation has arisen as a strategy to combat pathogen antigenic diversity. Indeed in HBV infection, maximal HLA variation appears to

Table 2 Gene polymorphisms associated with susceptibility to chronic hepatitis B

Gene/loci	Population	Sample size	P value	Reference
HLA B 08	Caucasian	563	0.03	[30]
HLA B 44-Cw 1601	Caucasian	563	0.02	[30]
HLA B 44-Cw 0501	Caucasian	563	0.006	[30]
HLA-DRB1 0301	Chinese	190	0.0074	[32]
HLA-DRB1 1301/2				[39]
HLA-DR6	Korean	1272	< 0.001	[33]
HLA-DQA1 0501	Chinese	190	0.0157	[32]
HLA -DQA1 0501	African American	91	0.05	[40]
HLA -DQB1 0301	African American	91	0.01	[40]
HLA-DQB1 0301	Chinese	190	0.0075	[32]
TNF-alpha-863 A	Korean	1400		[35]
TNF-alpha-238 GG genotype	Chinese	355	0.02	[36]
TNF-alpha-238 GG genotype	Chinese	455	0.02	[41]
TNF-alpha-857 CC genotype	Chinese	895	< 0.001	[34]
IFN-gamma A/A genotype		77		[42]
CTLA-4+6230 A			0.04	[37]
CCR5 59029 A allelic genotype	Chinese	377	0.002	[38]
ESR1 29 T/T genotype	Chinese	2318	< 0.001	[43]

have a direct protective effect, individuals with the most different alleles at class II HLA loci have the slowest HBV disease progression and the lowest mortality (a “heterozygous advantage”)^[51]. Conversely, lack of HLA diversity (a “homozygous disadvantage”) may increase the susceptibility to HBV infection among isolated communities^[52]. The extensive linkage disequilibrium across some HLA regions makes it difficult to localize specific disease-associated polymorphisms, although the HLA allelic association has allowed identification of critically pathogenic epitopes in some diseases^[59], which might act as potential vaccine candidates.

Disease associations involving loci outside the HLA region are also valuable in identifying the functional molecular basis underlying infectious disease resistance. For example, HIV uses various chemokine receptors as cofactors for CD4 binding to gain entry into human leukocytes. A functional polymorphism of the chemokine receptor CCR5, which is essential for HIV entry into macrophages, results in a truncated nonfunctional protein that confers highly significant protection against HIV susceptibility in the homozygous state and slows disease progression in heterozygotes^[53,54]. Chang *et al*^[38] have developed the association between CCR5 and HBV infection, though the biological process and significance in HBV infection need to be further studied.

Shortcoming of association studies in susceptible gene hunting

The number of studies seeking to identify genes that influence susceptibility to persistent HBV infection has greatly increased since we entered the “post-genomic” era. These studies are fuelled by the unlimited availability of

Table 3 Successful linkage analysis in infectious diseases

Diseases	Location of predisposing genes	Reference
<i>H pylori</i>	IFNGR1	[60]
Plasmodium falciparum	5q31-q33, MHC	[61-64]
Kala-azar	22q12, Imr2, Imr1	[65]
Tuberculosis	15q and Xq	[66]
Schistosoma mansoni	5q31-q33	[67]
Leprosy	10p13, 6q25	[68, 69]

SNPs, the relative ease of performing genotyping assays based on PCR technology, and the desire to identify major disease susceptibility gene(s). Literature is now littered with unreproducible genetic association studies that confuse the readers and have an understandable impact on the willingness of editors to accept further manuscripts for publication^[27]. *Nature Genetics* published an editorial in 1999 that set out a list of criteria for genetic association studies^[55]: plausible biological context, rigorous phenotypic selection (case selection), independent replication, rigorous genotyping, low *P* values, appropriate statistical analysis, and transmission disequilibrium test. Up to now, few candidate genes can fully meet the criteria.

Candidate gene-based association studies rely on having predicted the identity of the correct gene or genes, usually on the basis of biological hypotheses or the location of the candidate within a previously determined region of linkage^[56]. Even if these hypotheses are broad (for example, involving the testing of all genes in the insulin-signaling pathway), they will, at best, identify only a fraction of genetic risk factors for diseases in which the pathophysiology is relatively well understood. When the fundamental physiological defects of a disease are unknown, the candidate-gene approach is clearly inadequate to fully explain the genetic basis of the disease^[29]. In 2004, *Hepatology* editor appealed for less hypothesis-driven association studies that result in a negative or weak correlation^[27].

Linkage studies

Linkage is the tendency for genes and other genetic markers to be inherited together because of their location near one another on the same chromosome. Linkage studies classically seek to identify microsatellite markers that are inherited more commonly than expected by siblings who have the disease of interest (“affected sibling pairs”)^[57]. Genetic linkage analysis is a powerful tool to detect the chromosomal location of disease genes^[58]. A linkage study is to use a large number of families to look for regions of linkage to a disease, which suggest the presence of loci containing genes that may predispose to this disease. Linkage studies have the advantage of making no supposition about which genes might be involved in a disease, in that they merely identify stretches of chromosome around the microsatellite markers and can be used to examine the entire genome (a “whole genome screen”)^[58].

With the advent of new genetic markers and automated genotyping, genetic mapping can be conducted extremely

rapid^[28,59]. This approach has been successful in some infectious diseases (Table 3), but no report on such similar scans for HBV viral persistence is available. Recently a research team of Xi’an Jiaotong University has collected 327 HBV-infected subjects of 32 family pedigrees from a remote village (data not published), which makes it possible to find chromosome regions containing determinant(s) of persistent HBV infection. Their results will be reported soon.

CLINICAL IMPLICATION OF GENETIC STUDIES OF HBV INFECTION

Studies of the genetic determinants for HBV susceptibility can reveal fundamental data concerning the human immune system. The ultimate goal of such studies is the identification of critical immunologic mechanisms in the disease process to develop specific therapeutic interventions. As the precise immune deficiency is identified, it may be possible to “bypass” the identified immune deficiency with a specific therapy.

A specific genetic defect has been identified in rarer single gene defects, which may offer preconception genetic counseling to affected families. In complex diseases it might ultimately be possible to identify patients whose risk factors make them candidates for targeted therapies. Once the genotypic markers for a poor outcome of HBV infections are found, they in combination with rapid genotyping technology may allow more intensive therapies for those patients who are at the greatest risk of poor outcome and death^[70,71]. The potential to target drug treatment, both in terms of identifying patients most likely to benefit clinically and in terms of predicting those who are susceptible to either favorable or adverse pharmacologic outcome, is of great importance. It is conceivable that in the future our understanding of host genetics will largely influence our therapeutic response to HBV-infected patients and determine our choice of both preventive and curative interventions.

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TOPIC HIGHLIGHT

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Conventional therapy for Crohn's disease

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Abstract

Crohn's disease (CD) is a multifactorial disorder of unknown cause. Outstanding progress regarding the pathophysiology of CD has led to the development of innovative therapeutic concepts. Numerous controlled trials have been performed in CD over the last years. However, many drugs have not been approved by regulatory authorities due to lack of efficacy or severe side effects. Therefore, well-known drugs, including 5-ASA, systemic or topical corticosteroids, and immunosuppressants such as azathioprine, are still the mainstay of CD therapy. Importantly, biologicals such as infliximab have shown to be efficacious in problematic settings such as fistulizing or steroid-dependent CD. This review is intended to give practical guidelines to clinicians for the conventional treatment of CD. We concentrated on the results of randomized, placebo-controlled trials and meta-analyses, when available, that provide the highest degree of evidence. We provide evidence-based treatment algorithms whenever possible. However, many clinical situations have not been answered by controlled clinical trials and it is important to fill these gaps through expert opinions. We hope that this review offers a useful tool for clinicians in the challenging treatment of CD.

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Key words: Crohn's disease; Conventional treatment; Review; Therapy

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INTRODUCTION

Crohn's disease (CD) is a chronic bowel disease charac-

terised by a relapsing inflammatory process. It can affect any part of the gastrointestinal tract and is associated with discontinuous, transmural lesions of the gut wall. The current working hypothesis suggests that CD results from an aberrant immune response towards fecal bacteria in a genetically susceptible host^[1].

While medical treatment of the acute flare is successful in most patients, one of the most difficult tasks in general medicine is to treat complications such as strictures, abscesses, fistulae and chronic disease activity. In this review, we describe the conventional treatment of CD depending on different clinical situations, such as an acute flare, maintenance of remission, fistulizing or chronically active disease behaviour.

Apart from the below discussed medical and surgical treatment of CD, other factors including changes in lifestyle should be recommended. Herein, probably the most important aspect is smoking cessation. Smoking has shown to be a risk factor for CD relapse after medically or surgically induced remission^[2] and is associated with the need for higher doses of corticosteroids and immunosuppressants^[3]. Importantly, a prospective trial showed that only one year of smoking cessation leads to a more benign course of disease with a lower rate of relapses^[4]. This trial also showed that the ability to quit smoking clearly depended on the physician's role. So the conventional treatment of CD should start, if necessary, with convincing the patient to quit smoking.

ACTIVE DISEASE

Definition

The activity of CD can be assessed clinically, endoscopically or by other indices^[5]. The most established way is through the BEST activity index (CDAI), where symptoms and objective criteria such as anemia and body weight are included^[6]. Index values of 150 and below are associated with quiescent disease; values above that indicate active disease, and values above 450 are seen with extremely severe disease. In addition other diagnostic values such as blood sedimentation rate, C-reactive protein (CRP) and thrombocytes should also be taken into account. Endoscopic inflammatory evaluation, however, is not necessary in every exacerbation of the disease but might offer important information with respect to disease localisation. This is especially important for the use of topically acting agents such as budesonide in terminal ileal or right colonic CD. Exacerbation of CD through infectious agents should always be considered and

Table 1 Drugs for the treatment of CD

Drug	5-ASA (mesalamine or sulfasalazine)
Dosage	3.2-4 g/d
Indications	Mild to moderately active disease, postoperative maintenance
Important side effects	Headache, nausea and abdominal pain, often during treatment with sulfasalazine (in up to 45% of patients); thrombopenia; interstitial nephritis, pancreatitis;
Monitoring	Liver function, full blood count and especially renal function
Pregnancy	Suggested to be safe in conventional doses

excluded if possible.

In addition, the American College of Gastroenterology has defined the different disease activities in clinical practise as follows^[7]: mild to moderately active disease is defined as “ambulatory patients able to tolerate oral alimentation without manifestation of dehydration, toxicity (high fevers, rigors, prostration), abdominal tenderness, painful mass, obstruction or > 10% weight loss. In contrast, moderate to severe disease applies to patients that have failed to respond to treatment for mild to moderate disease or those with more prominent symptoms such as fever, significant weight loss, abdominal pain or tenderness, intermittent nausea or vomiting (without obstructive findings), or significant anemia. Severe disease refers to patients with persisting symptoms despite the introduction of steroids as outpatients, or individuals presenting with high fever, persistent vomiting, evidence of intestinal obstruction, rebound tenderness, cachexia, or evidence of an abscess.

5-ASA

No debate has been as longstanding and controversial as whether the use of 5-ASA containing drugs in CD is justified or not. Numerous studies regarding this aspect have been performed over the last 25 years. However, data from current studies do not clearly support either point of view. Different study designs and drug dosages have been used that make comparison of the results rather difficult.

Sulfasalazine is the original compound in this class consisting of 5-ASA linked by an azo-bond to sulfapyridine, which is split off in the colon. Therefore efficacy of sulfasalazine was expected to be limited to colonic disease. Furthermore up to 50% of patients are not able to tolerate sulfasalazine due to nausea, headache, vomiting and epigastric pain. These side effects are suggested to be caused by the sulfapyridine moiety. Therefore, other 5-ASA formulations (mesalamine formulations and the pro-drugs olsalazine and balsalazide) without sulfapyridine have been introduced into the market with different pharmacodynamic and pharmacokinetic profiles (Table 1). These different preparations are therefore suggested to be non-interchangeable.

Sulfasalazine: Sulfasalazine has been shown to be significantly better than placebo in randomized clinical trials in inducing remission in active CD^[8-10]. Subgroup analyses suggested that patients with only colonic disease seem to

benefit the most from sulfasalazine therapy^[8,9], whereas patients treated previously with prednisone failed to respond^[8]. Sulfasalazine has not shown to have steroid-sparing properties^[9,11]. Since 5-ASA was identified to be the active moiety in sulfasalazine, other 5-ASA containing formulations (such as mesalamine) have been tested in CD. **Mesalamine:** Different pharmacological preparations allow release of the active drug in different parts of the intestine. Therefore mesalamine, in contrast to sulfasalazine, may also be used in CD including small bowel CD. However, studies on the induction of remission in active CD with mesalamine yielded conflicting results. In total, six placebo-controlled trials with varying dosages of mesalamine have been performed to date. Two earlier studies did not detect a benefit of mesalamine over placebo in inducing remission^[12,13]. Tremaine and colleagues observed a significantly greater number of patients that responded (defined as either a decrease of CDAI \geq 70 or CDAI < 150), but this benefit was rather small (9 patients with mesalamine treatment *vs* 4 patients in the placebo group). However, no significant differences were found when clinical remission (defined as CDAI < 150) was analyzed^[14]. Singleton and colleagues conducted three different trials with mesalamine (Pentasa) that were recently combined in a meta-analysis although two of the three trials were never published in full^[15]. This analysis found a statistically significant benefit of mesalamine over placebo. However, this benefit was rather small (CDAI reduction of 18 points for the intention-to-treat-analysis).

In summary the clinical benefit of mesalamine in the treatment of active CD seems to be rather low. However, mesalamine is well tolerated and has a favourable side effect profile compared to sulfasalazine. The latter factor is probably the main reason why mesalamine is significantly used more often compared to sulfasalazine although data from randomized trials are in favor of sulfasalazine. Furthermore, many patients with mild to moderately active disease try a more harmless drug at first before taking corticosteroids.

Budesonide

The introduction of the topically-acting steroid budesonide has become a very potent alternative in the treatment of patients with CD located in the terminal ileum or right colon. Due to rapid metabolism by cytochrome P-450 enzymes in the liver, budesonide has less systemic bioavailability than systemic corticosteroids. A recent meta-analysis combined the data from 5 published studies investigating budesonide in comparison to placebo, 5-ASA and systemic corticosteroids^[16]. A significant advantage of budesonide in inducing remission was observed in comparison to placebo (odds ratio of 1.85) and mesalamine (odds ratio of 1.73). Accordingly, a patient is 73% more likely to achieve remission with budesonide than mesalamine. Corticosteroids induced remission even more often as compared to budesonide with an odds ratio of 0.87, but in patients with mild and moderate disease (CDAI 200-300), no difference in remission rates was found. Treatment with budesonide was associated with similar side effects compared to mesalamine and placebo. Importantly, fewer side effects, such as acne, moon face

Table 2 Drugs for the treatment of CD

Drug	Systemic corticosteroids (prednisone equivalent) or budesonide
Dosage	Corticosteroids: 30-60 mg/d or 1-1.5 mg/kg per day; Budesonide: 9 mg
Indications	Corticosteroids: moderate to severe disease. Budesonide: terminal ileal and right colonic disease in mild to moderate disease, low dose budesonide eventually for maintenance therapy
Important side effects	Weight gain, hypertension, fluid retention, myopathy, mood changes, infections, glaucoma, skin changes including acne, adrenal suppression. Long term side effects: osteoporosis, cataract, aseptic bone necrosis
Pregnancy	Lower doses seem to be relatively safe
Comments	Avoid long-term use

Table 3 Drugs for the treatment of CD

Drug	Azathioprine (6-mercaptopurine)
Dosage	2-2.5 mg/kg (1-1.5 mg/kg)
Indications	Maintenance, chronically active disease, steroid-refractory and steroid-dependency, fistulae, concomittant therapy with infliximab;
Important side effects	Pancreatitis, bone marrow suppression, allergic reactions, drug hepatitis, nausea, malaise, bacterial and viral infections; in patients intolerant to azathioprine due to gastrointestinal symptoms, 6-mercaptopurine is suggested (not in side effects such as pancreatitis and bone marrow suppression)
Monitoring	Liver function, lipase and full blood count biweekly for the first three months, if normal then every three months throughout therapy
Pregnancy	Should be avoided, although available studies suggest a potential use especially in patients where maintaining remission is essential
Comments	Entire therapeutic efficacy is observed mostly after 2-4 mo; consider testing for thiopurine methyltransferase (TPMT) genotypes to identify patients with high-risk of bone marrow suppression; consider metabolite monitoring for adequate dosing; ensure adequate birth control; allow 3 mo time before pregnancy or conceiving

and osteoporosis, were observed compared to systemic corticosteroids. The recommended dose of budesonide is 9 mg/d and should be tapered 3 mg every 2-4 wk unless a maintenance therapy with budesonide is suggested (see below).

Systemic corticosteroids

For moderate to severe CD, and especially if therapy with 5-ASA has failed, systemic corticosteroids are the treatment of choice (Table 2). Corticosteroids are fast and effective and induce remission in approximately 70% of patients. In active CD, corticosteroids have been shown to be superior to sulfasalazine, azathioprine and placebo^[8,9]. No dose finding studies have yet been performed. Reported doses range from 30 mg/d to 1 mg/kg per day, however most clinicians start with 60 mg/d, although it seems to be favourable to apply a body weight dependent dosage (1 mg/kg). Tapering should be performed

Table 4 Drugs for the treatment of CD

Drug	Methotrexate
Dosage	25 mg/wk i.m., if remission is achieved reduce to 15 i.m. (or s.c.)
Indications	Maintenance, chronically active disease, steroid-refractory and steroid-dependency, fistulae
Important side effects	Nausea, abdominal pain, diarrhea, stomatitis; hepatitis, liver fibrosis; hypersensitivity pneumonitis
Monitoring	Liver function and full blood count monthly for the first two months, if normal then every two months throughout therapy
Pregnancy	Strictly prohibited
Comments	Entire therapeutic efficacy is observed mostly after 2-4 mo; consider folic acid supplementation with 2.5-5 mg/d; ensure adequate birth control; allow 3 mo time before pregnancy or conceiving

according to improvement of clinical symptoms and is usually done in steps of 5-10 mg/wk. At lower dosages, tapering might be reduced to 2.5-5 mg/wk. Whether i.v. application has an advantage over oral in severe acute flares is not clear, although it is frequently used when oral treatment has not been effective.

Azathioprine/6-Mercaptopurine

The most commonly used immunomodulators are the thiopurines, 6-mercaptopurine and its prodrug azathioprine (Table 3). Numerous clinical trials studied the efficacy of these immunomodulators in active CD. The most convincing data were obtained in the early trial by Present and colleagues where 67% vs 8% of the patients in the 6-mercaptopurine group vs placebo, respectively, achieved remission^[17]. However, other trials did not observe a significant difference in the use of azathioprine compared to placebo^[8,18]. Despite these conflicting data, a meta-analysis reported an odds ratio of 3.09 favoring azathioprine/6-mercaptopurine therapy over placebo^[19] to induce remission. In addition, a recent Cochrane analysis reported an overall response in active CD of 54% vs 33% for azathioprine vs placebo, respectively^[20].

Thiopurines are slow acting drugs and an effect can be observed after 2-3 mo. Thus thiopurines are less frequently used to induce remission in an acute exacerbation but rather to maintain remission. However, they have been shown to have steroid sparing properties^[19,20] and furthermore the combination of prednisolone and azathioprine has shown to be superior over prednisolone monotherapy^[21]. Therefore it is suggested to add azathioprine to corticosteroids in severe CD.

Methotrexate

In the pivotal trial by Feagan and colleagues, methotrexate given intramuscularly 25 mg once a week was more likely to induce remission compared to placebo (Table 4). In addition, steroid-sparing properties were noted^[22]. However, side effects were more common with methotrexate therapy than with placebo. Other studies using low dose methotrexate did not show a significant benefit^[23,24]. In addition, no benefit was observed when high intravenous methotrexate was compared to oral

Table 5 Drugs for the treatment of CD

Drug	Metronidazole
Dosage	10-20 mg/kg
Indications	Mild to moderately active disease; fistulae (usually prolonged treatment)
Important side effects	Nausea, metallic taste in the mouth, coating of the tongue, peripheral neuropathy
Monitoring	See side effects
Pregnancy	Long term treatment not yet evaluated, short term treatment appears to be safe

Table 6 Drugs for the treatment of CD

Drug	Ciprofloxacin
Dosage	1-2 g/d
Indications	Mild to moderately active disease, fistulae
Important side effects	Taste disturbance, gastrointestinal events, tendopathies
Monitoring	Generally well tolerated, see side effects
Pregnancy	Probably safe

azathioprine^[25]. Like azathioprine/6-mercaptopurine, intramuscular methotrexate is only rarely used to treat an acute exacerbation of CD but is used more frequently in chronic active CD^[26]. Importantly, side effects with methotrexate, specifically liver dysfunction, are common and need to be monitored. In addition, methotrexate is contradicted during pregnancy and should be used very cautiously in women of child-bearing potential.

Antibiotics

Although antibiotics are frequently used to treat CD, this practice is not supported by strong evidence from randomized trials. However, increasing knowledge of the importance of mucosal bacteria for the pathogenesis of CD gives a good rationale for investigating antibiotic approaches^[27]. In addition, distinguishing an acute flare from an infectious gastroenteritis/colitis can be difficult. Thus antibiotics provide a therapeutic alternative, which might benefit both an acute flare and a gastrointestinal infection. However, further studies are warranted to establish the role of antibiotics in the treatment of CD and at this time they cannot be recommended as standard therapy.

Metronidazole: Metronidazole (20 mg/kg per day) has been shown to be superior over placebo in reducing the CDAI but not with respect to the induction of remission^[28] (Table 5). Furthermore, this benefit was only seen in patients with colonic or ileocolonic disease, whereas no benefit was found with disease location in the ileum. Similar findings were reported from another trial where few patients with colonic involvement showed an improvement^[29]. Another study reported no benefit *vs* placebo^[30]. Compared to sulfasalazine, a cross over study reported no difference in the first 4 mo. However, in the cross over design, patients switched to metronidazole showed an improvement of CDAI, whereas in the sulfasalazine group this was not the case^[31,32].

Table 7 Drugs for the treatment of CD

Drug	Infliximab
Dosage	5 mg/kg per infusion; usually started at wk 0, 2, and 6 and then repeated every 8 wk if necessary
Indications	Chronically active disease, steroid-refractory and steroid-dependency, maintenance, fistulae
Important side effects	Nausea, headache, abdominal pain, infections, sepsis; infusions reactions (early or delayed), reactivation of tuberculosis
Monitoring	Vital signs around infusion
Pregnancy	Unknown
Comments	Exclude tuberculosis before infusions, consider concomittant use of immunosuppressants (azathioprine) to reduce antibody formation

Ciprofloxacin: Ciprofloxacin is often used in a clinical routine, especially in combination with metronidazole (Table 6). Ciprofloxacin was significantly better compared to placebo in inducing remission in a smaller trial^[33] and was shown to be similarly effective compared to mesalazine^[34]. In contrast, corticosteroids resulted in higher rates of clinical remission compared to ciprofloxacin and metronidazole^[35]. In patients with chronically active disease on budesonide, the addition of metronidazole and ciprofloxacin was not superior over budesonide monotherapy, although in patients with colonic CD a trend towards a significant benefit was observed^[36].

Infliximab

Infliximab is a chimeric IgG1 monoclonal antibody against TNF- α (Table 7). Apart from inhibiting TNF- α , recent data suggest that the induction of apoptosis in T cells through infliximab might be an important mechanism of action^[37]. Infliximab has shown to be superior over placebo in inducing remission in patients with moderate to severe CD resistance to standard therapy^[38]. In this trial, after four weeks 33% of patients went into remission after one single infliximab infusion as compared to 4% of the patients given placebo.

Summary: Treatment of active CD

In mild and moderately active CD, 5-ASA or budesonide may be used as first line therapy, despite the limited efficacy of 5-ASA shown in randomized, placebo-controlled trials. The presently available budesonide preparations are only efficacious in disease primarily located within the terminal ileum or right colon. In non-responders, systemic corticosteroids should be used. Severe CD should be treated with systemic corticosteroids. If corticosteroids given orally do not lead to improvement, intravenous application should be considered since enteral absorption might be decreased due to severe intestinal inflammation. Enteral nutrition should also be added particularly in malnourished patients (see below chapter on nutrition for details). If an infectious complication is suspected, the additional therapy with antibiotics (e.g. ciprofloxacin plus metronidazole) might be beneficial. The combination of systemic corticosteroids and azathioprine is superior to prednisolone monotherapy and this combination might be beneficial in severe cases. In patients

refractory to corticosteroids, treatment with infliximab should be considered. Surgery might be necessary in patients with severe and refractory CD not responding to above mentioned strategies. Intravenous cyclosporin and tacrolimus should only be used in selected severe and refractory cases.

MAINTENANCE OF REMISSION

Maintaining a medically or surgically induced remission of disease is one of the most important but yet most difficult therapeutic goals in the treatment of CD. Maintenance therapy in CD is characterized as treatment with only a few available drugs, moderately high rates of efficacy and frequent side effects. In total, 40%-70% of CD patients will experience a symptomatic relapse in 1 year after a medically or surgically induced remission^[8,9]. Silverstein and colleagues reported that a surgically induced remission lasts a mean of 766 d whereas a non-surgically induced remission lasts only 120 d indicating that a surgically induced remission is more stable^[39]. It was frequently recommended that the indication for a relapse-preventing therapy should be based on the prospective risk of an individual patient to relapse. Although the estimation of risk for relapse, based on the phenotype or genotype, is still controversial, single well known risk factors like smoking, frequent relapses in the past, a chronic active disease *etc.* have been described. To stop smoking is a very important therapeutic goal^[2,40]. Systemic corticosteroids should not be used for maintaining remission due to lack of efficacy and severe long-term side effects. Since randomized, placebo-controlled trials suggest a different approach in medically or surgically induced remissions, we will handle them separately.

Medically induced remission

5-ASA: Numerous randomized, placebo-controlled studies, including four meta-analyses, have attempted to establish a role for 5-ASA in the maintenance of remission. Different study regimens and durations were performed and a substantial number of trials included only small numbers of patients. The two most recent meta-analyses failed to show a benefit for mesalamine over placebo in the maintenance of medically induced remission^[41,42]. However, the preferable side effect profile of 5-ASA, especially mesalamine compared to azathioprine/6-mercaptopurine or methotrexate, is probably the reason why mesalamine is still used frequently to maintain a medically-induced remission. Many clinicians therefore try to maintain remission with mesalamine at least one time, especially in young women of childbearing potential. In addition many patients are in favour of trying a rather harmless drug at first for long-term therapy.

Azathioprine/6-Mercaptopurine: Azathioprine/6-mercaptopurine is the treatment of choice for patients with high risk of relapse. The effectiveness of azathioprine has been described in a recent meta-analysis including five randomized, placebo-controlled trials. In addition, a steroid-sparing effect was observed^[43]. No clear direction has been given as to when to start the treatment with azathioprine/6-mercaptopurine. The following indications are most

commonly accepted: frequent flares (more than two per year), chronically active disease, and steroid dependence (e.g. if two attempts of tapering steroids have failed). The thiopurines are slow acting drugs and an effect is usually observed after 2-3 mo with approximately 90% of patients responding within the first 4 mo^[17].

An earlier open study suggested that azathioprine is no longer effective after 3.5 years^[44]. In contrast, the same group reported, in a very recent placebo-controlled trial, that azathioprine is still effective with prolonged use^[45]. However, a small increase in the frequency of malignancy, especially lymphoma, cannot be excluded in the long term treatment with azathioprine/6-mercaptopurine^[46,47]. This must be weighed against the improved quality of life due to both drugs for patients with CD.

Methotrexate: The potential of methotrexate to induce remission was investigated in a study by Feagan and colleagues. Herein the patients who had achieved remission after weekly 25 mg intramuscularly were randomized to 15 mg methotrexate or placebo. Methotrexate was found to be significantly better than placebo in maintaining remission^[26]. However, side effects were more significant than placebo. Methotrexate has not been studied in surgically or medically induced remission by other drugs (e.g. corticosteroids). In summary, methotrexate is suggested to be the alternative to azathioprine/6-mercaptopurine in the maintenance of remission. It has also shown to have steroid-sparing properties with the mean time to respond at about 2 mo.

Budesonide: Lower doses of budesonide (3 or 6 mg) have also been studied for their potential to be effective in the maintenance of remission. Although earlier meta-analyses have not shown that budesonide was superior over placebo^[16,48], a recent randomized, placebo-controlled trial found a trend towards a longer quiescent disease in budesonide treated patients compared to placebo^[49]. In this trial, no significant difference in total adverse events or corticosteroid-associated events was demonstrated between placebo and budesonide. In addition, a very recent paper by Sandborn and colleagues combined the data of four double-blind, placebo-controlled trials with identical protocols analyzing the efficacy of 6 mg budesonide. Budesonide was shown to be effective for prolonging the time to relapse and for significantly reducing the rates of relapse at 3 and 6 mo but not at 12 mo. Herein no difference in the frequency of adverse events and glucocorticosteroid associated side effects between budesonide and placebo was found^[50]. Thus, the current data suggest that budesonide at a dosage of 6 mg seems to have the effect of prolonging remission in CD in terminal ileal or right colonic disease. Budesonide might thus offer a potential alternative in the maintenance of a medically-induced remission, especially in steroid-dependent patients.

Infliximab: Two studies have shown that infliximab is effective in maintaining remission in CD^[51,52]. In the Accent I trial, infliximab was shown to be superior over placebo in the maintenance of remission in CD patients that responded to one single infusion of infliximab. Herein, about 20% of patients in remission after the first infusion of infliximab were maintained in remission for one year with repeated infusions every eight weeks^[52]. Infliximab

was also shown to have steroid-sparing properties. Repeated infusions of infliximab should thus be considered for chronically active or steroid-dependent patients where standard immunosuppressants are not effective or where surgical interventions are not considered. However, repeated infusions of infliximab are costly and data on long-term safety, including the occurrence of malignancies, are limited. Infliximab has been shown to lead to mucosal healing, which was associated with reduced surgical interventions and lower hospitalization rates^[53]. However, at this time it is debated whether mucosal healing is an important goal in CD therapy. Further studies are warranted regarding this matter. The development of antibodies against infliximab is frequently found and is associated with reduced efficacy and increased numbers of infusion reactions. The concomitant use of immunosuppressants has been shown to reduce the incidence of antibody formation^[54].

Summary: Maintenance after medically induced remission

After a medically-induced remission, maintenance therapy should be initiated based on the individual situation. No medical therapy may be considered in patients with low risk of relapse. However, in patients with high risk for relapse (frequent relapses, colonic involvement and severe disease behaviour), therapy with azathioprine or 6-mercaptopurine should be initiated. In patients with terminal ileal or right colonic disease, low-dose budesonide might offer an alternative especially in steroid-dependent patients. In patients who are not responding or are intolerant to azathioprine/6-mercaptopurine, therapy with methotrexate may be used. If not successful, patients should be considered for maintenance treatment with infliximab.

Postoperative CD (surgically-induced remission)

About 75% of CD patients will require surgery within the first 20 years after the onset of symptoms^[55,56]. In addition, recurrence rates after surgical resection are high: after the first resection, up to 80% of patients show an endoscopic recurrence within the first year although most patients are not symptomatic^[55-57]. Furthermore, up to 20% have clinical symptoms and 5% require another surgical intervention within the first year. After 5 years, about 50% of patients have a clinical relapse. Systemic corticosteroids and budesonide are not effective in preventing postoperative relapse^[58-61], whereas methotrexate, ciprofloxacin and infliximab have not been studied for this indication. Various risk factors for postoperative recurrence have been described but most of these risk factors have not been studied in a prospective manner. Currently smoking is the most consistently described risk factor for postoperative relapse^[40,62]. In addition, Rutgeerts and colleagues showed that preoperative disease activity and endoscopic lesions at the neoterminal ileum within the first year after surgery are also associated with higher risk for postoperative recurrence^[57]. In addition, a recent study suggested that CD patients with CARD15 mutations have a higher risk of postoperative relapse compared to patients without mutated CARD15. Thus genotyping for CARD15 mutations might offer a potential alternative to identify patients with high risk of postoperative relapse^[63]. Further

studies are warranted to consider this approach.

5-ASA: As opposed to the controversial discussion about the efficacy of 5-ASA in the treatment of CD, the results on the prevention of postoperative recurrence are quite solid. Camma and colleagues described in a meta-analysis a risk reduction of 13.1% by mesalamine treatment compared to placebo^[41]. A more recent placebo-controlled trial reported that mesalamine did not significantly affect the postoperative course of CD, but some relapse-preventing effect was found in patients with isolated small bowel disease^[64]. In summary, 5 ASA is the only treatment with an evidence-based relapse preventing effect after a surgically induced remission and is therefore recommended according to recent guidelines^[65].

Azathioprine/6-Mercaptopurine: The two largest studies regarding the effect of azathioprine/6-mercaptopurine to prevent postoperative recurrence were recently published. In the first trial, Hanauer and colleagues compared 6-mercaptopurine at the low fixed dose of 50 mg/d to mesalamine 3 g/d and placebo after ileocolic resection^[66]. There was a significant benefit of 6-mercaptopurine compared to placebo in preventing clinical and endoscopic recurrence over two years. However, this study has been criticized since it was underpowered and also had a high dropout rate of patients. Ardizzone and colleagues observed no benefit of azathioprine at standard dosing (2 mg/kg) in preventing clinical relapse after two years in comparison to mesalamine^[67]. In summary, although none of these studies offer robust data to support the use of azathioprine/6-mercaptopurine in the prevention of postoperative recurrence, many clinicians use these drugs for this indication.

Antibiotics: In a randomized, placebo-controlled trial a significant decrease was observed in the incidence of severe endoscopic recurrence with metronidazole treatment as compared to placebo after ileal resection^[68]. In addition, metronidazole therapy statistically reduced the clinical recurrence rates at 1 year. Metronidazole is still only rarely used on this occasion since long term intake is not tolerated by most patients due to side effects such as metallic taste, nausea and peripheral neuropathy. Ciprofloxacin has not been studied in a randomized, placebo-controlled trial regarding the prevention of postoperative recurrence. Rifaximin, which is a non-absorbable drug with good tolerability covering most Gram-positive and Gram-negative bacteria, might offer a very promising alternative since long term application is tolerated much better^[69]. At the moment, although frequently used in clinical practice, none of these antibiotics will be considered standard therapy until more controlled trials provide clear results.

Rutgeerts and colleagues investigated the efficacy of ornidazole, a nitroimidazole antibiotic, for the prevention of clinical recurrence after curative ileocolonic resection in a recent placebo-controlled trial. They found that ornidazole significantly reduced the clinical and endoscopic recurrence rate at 1 year compared to placebo. However, significantly more patients in the ornidazole group dropped out of the study because of side effects. In summary, these data indicate that ornidazole might offer a therapeutic alternative in preventing postoperative recurrence^[70].

Summary: Treatment of postoperative CD

No standard treatment algorithm prevents postoperative relapse. Despite the controversial discussion on its efficacy, mesalamine over a period of two years is recommended as the treatment of choice in the prevention of postoperative relapse. However, many patients who undergo surgical resection have already been treated with mesalamine so that alternative regimes should be initiated. Although robust data are lacking, most clinicians use azathioprine/6-mercaptopurine at standard dosing in patients with higher risk of postoperative relapse. To estimate the risk of clinical relapse the diagnosis of endoscopic lesions at the anastomosis 6 mo after resection may be used. Azathioprine/6-mercaptopurine may be started if severe or moderate lesions at the anastomosis are found^[71]. Although this regime has never been studied in a randomized trial, it seems to be a reasonable approach. Antibiotics, such as metronidazole or ornidazole, might offer a potential alternative although the long term use is limited due to side effects. In addition, prospective studies investigating infliximab in this setting are warranted.

COMPLICATIONS IN CD**Fistulizing disease behaviour**

The treatment of fistulizing CD remains probably the most difficult clinical challenge. Treatment is complicated since very few drugs have proven efficacy whereas most agents used in CD therapy (5-ASA, systemic corticosteroids, budesonide) are ineffective. Fistulae are reported to occur in up to 50% of patients after 20 years of disease^[72]. Especially enterocutaneous and enterovaginal fistulae have a severe impact on the quality of life of CD patients. Enterovesical fistulae require surgical intervention due the potential development of an urosepsis. Perianal fistulae are the most common form and are often complicated by an abscess where surgical drainage must be performed. Since complete long term closure of fistulae cannot be achieved in many patients with the available therapies, reduction of fistula drainage and closure of part of the fistulae have been accepted therapeutic goals. Apart from medical treatment approaches as discussed below, surgical interventions such as fistulotomy and insertion of non-cutting setons should be part of the management. A close cooperation between the gastroenterologist and the surgeon is required.

Azathioprine/6-Mercaptopurine: Robust data summarized in the meta-analysis by Pearson^[19] show a positive effect of azathioprine/6-mercaptopurine on fistula closure with an odds ratio of 4.44 (CI 1.50-13.20). Thus azathioprine/6-mercaptopurine is the basis of long-term treatment of fistulae.

Methotrexate: No randomized trial has been performed using methotrexate to investigate the healing of fistulae. However, retrospective data showed complete or partial response in 56% (9/16) of patients^[73]. Methotrexate might thus be considered as the alternative agent to azathioprine/6-mercaptopurine.

Antibiotics: A small uncontrolled study reported a clinical response to metronidazole in 20 out of 21 patients and complete healing after maintenance treatment in 10 out of

18 patients. A follow-up study demonstrated that dosage reduction was associated with exacerbation of fistulae in all patients and healing was again achieved if the drug was reintroduced^[74]. Ciprofloxacin alone showed an improvement in 7 out of 10 patients treated with up to 1.5 g over three months^[75]. Although controlled clinical trials are lacking, the combination of metronidazole and ciprofloxacin is often initiated.

Infliximab: Infliximab offers robust data from randomized, placebo-controlled trials in the treatment of enterocutaneous fistulae. In the first trial by Present and colleagues, three infusions of infliximab at 0, 2, and 6 wk resulted in complete healing of enterocutaneous fistulae in 55% of patients compared to 13% in the placebo group^[76]. Data from the ACCENT 2 trial showed that infliximab maintained healing of enterocutaneous fistulae in 36% patients who responded to the initial three infliximab infusions^[77]. However, it was shown that healing of fistulae needed repeated infusions, which is similar to the experiences observed in a clinical routine. In summary, data from controlled clinical trials suggest that infliximab might be the most potent drug in the treatment of CD fistulae. Three infusions with a dose of 5 mg/kg at wk 0, 2, and 6 are recommended as standard for the treatment of fistulizing CD.

Cyclosporin A: Cyclosporin A offers an effective alternative treatment for CD fistulae. There are numerous uncontrolled trials that describe a mean initial response in 83% of patients with discontinuation of treatment leading to frequent relapses (reviewed in ref. [78]). However, cyclosporin A toxicity can be dramatic, including renal failure, and thus application should be performed only in centers with expertise. Continuous infusions of 4 mg/kg per day is required and concentrations of 300-400 ng/mL should be maintained^[78]. Dosing can be switched to oral if patients respond to intravenous cyclosporin.

Tacrolimus: A recent placebo-controlled trial showed that tacrolimus at a dose of 0.2 mg/kg was more effective than placebo in improvement of fistulae (defined as closure of \geq 50% of draining fistulas). However, no difference was observed with respect to fistula remission as defined by closure of all fistulas and maintenance of that closure for at least 4 wk. In addition, adverse events such as headache, increased serum creatinine levels, and insomnia were found significantly more often in the tacrolimus group^[79].

Summary: Treatment of fistulizing CD

No standardized treatment algorithm exists in the medical treatment of fistulizing CD. Importantly, effective management requires good collaboration between the gastroenterologist and the surgeon in both simple and complex fistulae. Azathioprine/6-mercaptopurine are the basis of fistulae treatment. Antibiotic combination therapy, preferable with metronidazole and ciprofloxacin, can be considered over a period of 2-3 mo especially if an abscess might be suspected to occur. In patients with complex fistulae including underlying rectal inflammation not improving from above mentioned strategies, a three dose therapy regimen with infliximab should be applied. If patients respond, therapy with azathioprine and infliximab might be necessary to maintain fistula healing. In refractory cases, therapy

with cyclosporine and tacrolimus should be considered.

Chronic active disease

Various definitions of chronic active disease exist and thus results from clinical trials in this complicated group of patients are rather difficult to interpret. The German consensus conference on the treatment of CD describes chronic active disease as the persisting or recurrent occurrence of symptoms over more than 6 mo despite standardized therapy^[65]. Patients with chronic active disease should thus be treated first with azathioprine/6-mercaptopurine or alternatively with methotrexate. If patients do not respond or are intolerant to these approaches, infliximab should be given. Due to severe long-term side effects, systemic corticosteroids should be avoided. 5-ASA is not effective in chronically active CD.

Steroid-dependent disease

Steroid-dependency is a frequently observed phenomenon in CD and it is defined as the need for corticosteroids to maintain a patient in stable remission after two unsuccessful attempts to withdraw steroids within the last six months. About 28%-44% of patients will become steroid-dependent after an initial course of corticosteroids^[80,81]. Long term use of corticosteroids should be avoided due to severe side effects such as osteoporosis, diabetes and hypertension. Prophylaxis of osteoporosis with calcium and vitamin D should be applied. Similar to patients with chronic active disease, azathioprine/6-mercaptopurine is the treatment of choice and methotrexate is the alternative agent to avoid long term steroid therapy. Two meta-analyses reported a steroid-sparing effect for azathioprine^[20,43] and the same properties were observed for methotrexate^[26]. In addition, infliximab has also been shown to have steroid-sparing properties and thus should also be considered as an alternative^[52].

Steroid-refractory disease

Patients with persisting clinical activity under continuing therapy with corticosteroids at a dose greater than 1 mg/kg per day are described as steroid-refractory. This clinical situation occurs in about 20%-30% of patients treated with corticosteroids^[8,9,80]. Only a few drugs have been tested in this situation: azathioprine/6-mercaptopurine and methotrexate have shown to be effective in steroid-refractory patients^[20,26,43]. In addition, infliximab offers a therapeutic alternative^[52]. However, if medical therapy fails in severe cases, surgical interventions such as colectomy might be necessary.

Gastroduodenal CD

Symptomatic involvement of stomach and duodenum is a rare phenomenon observed in about 4%-5.5% of patients^[82,83]. Endoscopic and histologic involvement might be found in up to 40% of patients^[84-86]. Due to the low frequency of patients with symptomatic gastroduodenal involvement, however, randomized, placebo-controlled trials are not available. Combination therapy with high dose acid suppression (proton pump inhibitors) and standard therapy of CD are usually used. Corticosteroids^[87], azathioprine^[88,89], and infliximab^[90] have been reported to

be effective in selected patients. However, many patients with obstructive symptoms caused by strictures will have to undergo surgical interventions such as gastroduodenal or gastrojejunal bypass, even performed laparoscopically. Gastroduodenal bypass has been reported to result in a good outcome in up to 87% of patients^[91].

Fibrostenotic disease behaviour

CD is often complicated by fibrostenotic strictures that can be located within the whole gastrointestinal tract. Strictures can remain clinically asymptomatic over years until the intraluminal caliber causes obstruction. However, it is often difficult to differentiate between an inflammatory or fibrostenotic stricture. Ultrasound and MRI with the possibility to visualize mucosal blood flow are helpful in differential diagnosis. Before initiating surgical interventions, many clinicians try at least one attempt of medical treatment for strictures suggested to have an inflammatory component. Corticosteroids are most commonly used in this clinical situation. Fibrostenotic strictures will not respond to medical therapy. Endoscopic balloon dilatations, stricturoplasty or resections are required in most cases.

ROLE OF NUTRITION IN CD

Prevention and treatment of malnutrition

During an acute flare of CD, undernutrition with weight loss, protein deficiency and specific deficiencies in vitamins, minerals and trace elements are commonly found. Malnutrition is mainly caused by anorexia, increased intestinal losses and systemic inflammation. In children and adolescents a decrease in growth velocity may occur, secondary to inadequate nutrition and steroid therapy. The relevance and extent of these deficiencies vary according to the site and extent of the diseased intestine as well as disease activity. In active CD, an improvement in nutritional status cannot be achieved by nutritional counselling alone but oral nutritional supplements or tube feeding leads to improvement of the nutritional status^[92,93]. Both malnutrition and growth retardation require enteral nutrition (EN).

The use of oral nutritional supplements or tube feeding should also be taken into account in the perioperative setting. An increased frequency of postoperative complications has been shown in undernourished patients with CD^[94], with undernutrition being defined as weight loss and/or plasma albumin levels below 35 g/L. Although specific data concerning the effect of perioperative nutrition in CD are lacking, there is a considerable body of evidence on the effect of perioperative nutrition in general gastrointestinal surgery and preoperative nutritional support is therefore recommended in malnourished patients^[95]. A prospective study showed that a preoperative oral supplementation with a formula enriched with arginine, omega-3 fatty acids, and RNA was associated with reduced postoperative infections and shorter lengths of hospital stay^[96]. Supplementation of specific deficiencies may be crucial. Iron deficiency is most common and should be treated with oral or i.v. iron supplements. Vitamin D and calcium should be supplemented in patients

on steroid therapy and patients treated with sulfasalazine are at risk to develop Vitamin B12 deficits.

Treatment of active disease

EN is also effective in the treatment of an acute flare in CD with approximately 60% of all patients reaching remission. In children, active disease frequently leads to growth retardation and enteral nutrition is therefore the treatment of choice. In adults, however, treatment with corticosteroids is more effective as shown by a recent meta-analysis^[97]. Enteral nutrition as sole therapy for acute CD is indicated mainly when treatment with corticosteroids is not feasible; e.g. due to intolerance or refusal. Combined therapy (enteral nutrition and drugs) is indicated in undernourished patients as well as in those with inflammatory stenosis of the intestine. If active CD is treated with systemic corticosteroids in combination with EN and supplementary EN is continued after the active phase, it prolongs the relapse free interval^[98].

Total parenteral nutrition is no better than enteral nutrition in the therapy of active CD and should therefore be restricted to patients with a contraindication to or intolerance of enteral nutrition^[99]. EN in subileus and high grade stenosis does require special caution. A documented stenosis however is not a contraindication to EN *per se*^[100].

EXTRAIESTINAL MANIFESTATIONS

CD is much more than a bowel disease since it can affect almost every other organ of the body. We will describe only briefly the most common extraintestinal manifestations (EIMs) and the recommended therapeutic approaches. The treatment of most extraintestinal manifestations has not arisen from randomized clinical trials but more from experiences and case reports and thus remains often nonempirical. With respect to all EIMs, a collaboration with rheumatologists, dermatologists and especially ophthalmologists should be part of the therapeutic regimen. The basis of treatment of EIMs is to obtain remission since it will positively affect the course of the particular extraintestinal manifestation, especially if symptoms occur parallel to exacerbation of the disease.

Arthritis

Joint involvement is the most frequently found extraintestinal manifestation in CD, which can be separated into axial and peripheral involvement. Peripheral involvement can be subdivided into a pauciarticular, large joint arthropathy, and a bilateral symmetrical polyarthropathy^[101]. Axial involvement can result in sacroiliitis or ankylosing spondylitis. Placebo-controlled trials have shown that sulfasalazine is effective in the treatment of ankylosing spondylitis^[102,103]. Furthermore physiotherapy is important. A low dose of corticosteroids (usually no more than 10 mg/d) can be a therapeutic option. Nonsteroidal anti-inflammatory drugs (NSAIDs) and COX-II-inhibitors might lead to pain relief but should be avoided since they might exacerbate CD. Many patients need analgetics to control symptoms. The use of tramadol or metamizol is preferable. Due to the experiences with rheumatoid arthritis, methotrexate might be offered as an

alternative. In the same respect, infliximab has shown to be very effective^[104,105].

Erythema nodosum, pyoderma gangrenosum, ocular involvement, PSC

Erythema nodosum is the most common skin manifestation in conjunction with active CD and usually responds to therapy with corticosteroids. Severe or refractory cases have been shown to respond to infliximab^[106]. In pyoderma gangrenosum, corticosteroids are the treatment of choice, even applied by the intravenous route in refractory cases. Topical therapy should be considered as an adjuvant to systemic therapy. However, a recent study reported healing of pyoderma gangrenosum after infliximab treatment in all 13 patients^[107]. These results suggest that infliximab might be considered as the treatment of choice for pyoderma gangrenosum, especially in refractory cases. An ocular manifestation such as iridocyclitis or anterior uveitis should be treated with topical steroids and cycloplegics. A case with improvement of uveitis after infliximab treatment was recently reported^[108]. Considering primary sclerosing cholangitis (PSC), although more frequently seen in ulcerative colitis, an earlier study showed that ursodeoxycholic acid (UDCA) at a dose of 10-15 mg/d can result in significant liver enzyme improvement^[109]. However, a recent 5-year, placebo-controlled trial of high-dose UDCA (17-23 mg/d) failed to show benefit for UDCA on survival or the prevention of cholangiocarcinoma in PSC^[110]. Taking all published studies into consideration, Olsson and colleagues conclude that there is, if at all, only a very limited effect of UDCA in PSC. PSC is associated with the occurrence of cholangiocarcinoma where liver transplantation seems to be the only curative approach.

CONCLUSION

Based on the currently available data from randomized, placebo-controlled trials, including meta-analyses, we describe the conventional treatment of Crohn's disease. This conventional approach suggests a step-up approach usually in the order of 5-ASA, corticosteroids, immunosuppressants and usually infliximab in refractory or severe cases including fistulizing disease behaviour. In contrast, a more aggressive form of treatment (bottom-down) has been recently proposed. This regimen starts out early at diagnosis of CD with the combination of biologicals (infliximab) in combination with immunosuppressants (azathioprine). Studies are warranted to elucidate the role of this new therapeutic approach in comparison to the standard therapy algorithms. Furthermore the value of mucosal healing and its effect on the course of CD, including its potential to reduce complications, surgical interventions and hospitalisation rates, should be evaluated in upcoming studies.

The past years have resulted in enormous new insights into the pathophysiology of CD with respect to molecular genetics, mucosal bacteria and immunology. Now it is time to translate these findings into newer therapeutic concepts. Numerous agents, especially biologicals, have been tested but most of them have not been introduced

into the market due to low efficacy or severe side effects. Apart from infliximab, other TNF α -antagonists, such as adalimumab or CDP870, might offer a potent alternative in the future. However, apart from evidence-based medicine, CD therapy will always be an individualized therapy. In addition, many patients construct their own therapeutic regimen, especially after long term disease. Such approaches might be effective in individual situations, although they do often not stand the criteria of evidence-based medicine. Moreover, many clinical situations are complex and might never have been studied in randomized, placebo-controlled trials. Therefore, the treatment of CD frequently requires individual decisions and creativity despite a very good basis of evidence-based therapies.

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Etiopathogenesis of inflammatory bowel diseases

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Abstract

Theories explaining the etiopathogenesis of inflammatory bowel disease (IBD) have been proposed ever since Crohn's disease (CD) and ulcerative colitis (UC) were recognized as the two major forms of the disease. Although the exact cause(s) and mechanisms of tissue damage in CD and UC have yet to be completely understood, enough progress has occurred to accept the following hypothesis as valid: IBD is an inappropriate immune response that occurs in genetically susceptible individuals as the result of a complex interaction among environmental factors, microbial factors, and the intestinal immune system. Among an almost endless list of environmental factors, smoking has been identified as a risk factor for CD and a protective factor for UC. Among microbial factors, no convincing evidence indicates that classical infectious agents cause IBD, while mounting evidence points to an abnormal immune response against the normal enteric flora as being of central importance. Gut inflammation is mediated by cells of the innate as well as adaptive immune systems, with the additional contribution of non-immune cells, such as epithelial, mesenchymal and endothelial cells, and platelets.

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Key words: Inflammatory bowel disease; Chronic inflammation; Mucosal immunity; Innate immunity; Adaptive immunity; Environment; Commensal flora

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INTRODUCTION

It is fair to state most disease entities that still pose major clinical and therapeutic challenges are ones where the exact etiology remains obscure and the mechanisms of tissue injury appear to be exceedingly complex. This certainly seems to be the case for the two main forms of inflammatory bowel disease (IBD); i.e., Crohn's disease (CD) and ulcerative colitis (UC). It is now clear that CD and UC represent two distinct forms of chronic inflammation of the gastrointestinal tract and, as such, have different causes and different pathogenic mechanisms. Still, the factors underlying the appearance of both CD and UC are roughly the same, and include a temporal association with progressive changes in the environment, an intrinsic genetic predisposition, the existence of a rich enteric flora, and an abnormal immune reactivity which is ultimately responsible for damaging the gut and causing clinical manifestations. Even though the categories of underlying factors are roughly the same, there are variations in each category as well as differences in how the underlying factors interact. The end result is two related but distinct disorders named CD and UC. In this review, differences and similarities of the etiopathogenic factors in each form of IBD will be briefly illustrated and discussed.

ENVIRONMENTAL AND GENETIC FACTORS

A remarkable change in the types of diseases affecting humans has occurred during the last century, most remarkably so in the Western world. The most common illnesses responsible for morbidity and mortality have shifted from infectious to chronic inflammatory and neoplastic diseases. This shift has been best documented in Western countries^[1], but the same phenomenon is now occurring in other parts of the world. The emergence of chronic autoimmune and inflammatory diseases, including IBD, throughout the world is closely linked to social and economical progress. This was initially noted in Northern Europe and North America but, after the Second World War, the same phenomenon occurred in the rest of Europe, Japan and South America. Most recently, the emergence of IBD is also being observed in the Asian Pacific Region^[2].

The "hygiene hypothesis" has been proposed as the probable underlying reason for the switch from infectious to chronic inflammatory diseases, and it postulates that there has been a fundamental lifestyle change from one with high microbial exposure to one with low microbial

exposure^[3]. A relative lack of microbial antigens early in life would lead to a less educated and weaker immune system, not equipped to properly handle new challenges later on in life and generating an ineffective immune response that is prolonged because it is powerless to eliminate the offending agent.

There are innumerable environmental modifications that can be ascribed to the hygiene hypothesis, including better housing, safer food and water, improved hygiene and sanitation, vaccines, the widespread use of antibiotics, lack of parasites, fewer infections, and better but selective nutrition. While contributing to the progressive decline of infectious diseases, at the same time these changes may have contributed to create a surge in allergic and autoimmune diseases^[4]. A variety of environmental factors are considered risk factors for IBD, including smoking, diet, drugs, geography and social status, stress, the enteric flora, altered intestinal permeability and appendectomy^[5]. Among them, cigarette smoking is the strongest example of the influence of the environment on IBD. Remarkably, smoking has a completely opposite effect on CD compared to UC, indicating that distinct pathogenic mechanisms underlie each form of IBD^[6]. Smoking is a recognized risk factor for CD, increasing the frequency of disease relapse and need for surgery, and its discontinuation improves the disease course^[7]. Cessation of smoking, however, increases the risk of UC, suggesting a protective role in this form of IBD^[8]. Other environmental agents associated with IBD are oral contraceptives and nonsteroidal anti-inflammatory drugs (NSAIDs). These agents have also been investigated as having a cause-and-effect relationship with CD or UC. A direct causal relationship has not been found, but women taking oral contraceptives have twice the risk of developing CD than those not taking contraceptives^[9]. In the case of NSAIDs there is a clear association with IBD, and patients in clinical remission have a higher risk of relapse if they use NSAIDs^[10].

Although the epidemiological evidence linking environmental factors to IBD is fairly solid, it is widely believed that no environmental factor alone can directly cause CD or UC, and an intrinsic disease predisposition must also be present. Such predisposition depends on genetic susceptibility, and a number of established or potential susceptibility genetic loci have been identified in IBD. This topic will be discussed in greater depth in another chapter of this issue of World Journal of Gastroenterology.

MICROBIAL FACTORS

Pathogens

It is possible that classical infectious agents are the cause of IBD, but current evidence supporting this hypothesis is rather weak. Over the years, several microorganisms, such as *Listeria monocytogenes*, *Chlamydia tracomatis*, *Escherichia coli*, *Cytomegalovirus*, *Saccharomyces cerevisiae*, as well as others, have been proposed as having an etiologic role. In particular, *Mycobacterium paratuberculosis* as the agent of CD has received and continues to receive considerable attention. This bacterium is the cause of Johne's disease, a chronic granulomatous ileitis in ruminants that closely resembles

CD. *M. paratuberculosis* was initially isolated from a few CD tissues^[11], but follow up studies trying to confirm its presence by histological examination, attempts to culture it from tissue homogenates, search for its genome in intestinal tissues with highly specific probes, and assessment of serum antibodies have all yielded conflicting or inconclusive results. Moreover, controlled trials have failed to show a beneficial effect of antituberculous therapy in CD patients^[12]. One of the last bacteria to be linked to CD is an adherent-invasive strain of *E coli* which is specifically associated with ileal CD^[13], but its potential etiologic role, if any, remains unclear.

The finding of paramyxovirus-like particles in CD endothelial granulomas led to the suggestion that CD could be a form of chronic vasculitis caused by the persistence of the measles virus in the mucosa^[14]. Based on epidemiological and serologic data, an association between perinatal measles and an increased probability to develop CD was hypothesized^[15], but subsequent studies failed to confirm this association. Importantly, the overall decline of measles infection accompanied by the concomitant rise of CD during the last few decades speaks against an etiologic role of measles in CD.

Commensal bacteria

In contrast to the dwindling evidence that CD or UC are infectious diseases, evidence continues to mount that the indigenous commensal flora of the gut is the target of the immune response in IBD^[16]. A large body of data from animal models of IBD indicates that the normal enteric flora is needed to develop experimental colitis. In fact, gut inflammation only arises in animals kept in a conventional but not a germ-free environment^[17], supposedly because an immune response directed against enteric bacteria is essential to disease pathogenesis^[18]. Thus, the paradigm "no bacteria, no colitis" was created to underscore the central role of the intestinal microbiota in IBD pathogenesis. This paradigm is supported by a variety of clinical observations in IBD patients. There is an increased number of bacteria in close contact with the mucosa in IBD patients^[19]; IBD lesions occur preferentially in segments with the highest concentrations of bacteria (the ileo-cecal valve and the colon); surgical diversion of the fecal stream prevents reappearance of CD whereas restoration of the fecal flow induces disease recurrence^[20]; modulation of the enteric flora with antibiotics and probiotics attenuates inflammation. In addition, pouchitis develops in a considerable proportion of UC patients, and is associated with a dysbiosis caused by the contact of the once near sterile small bowel mucosa with a rich colon-like flora repopulating the pouch soon after proctocolectomy^[21].

Finally, most IBD patients show an enhanced systemic and mucosal immunological reactivity against gut bacterial antigens. Among these, based on serum antibody titers, bacterial flagellin has been recently reported as a dominant antigen in CD^[22], apparently defining a population of patients with complicated CD^[23]. It has been proposed that this immune reactivity is the consequence of a 'loss of tolerance' towards the autologous enteric flora, resulting in an inappropriate immune response in the mucosa which is manifested by the chronic inflammatory process typical

of CD and UC^[24]. Under normal circumstances there is an intimate interaction between commensal intestinal bacteria and the immune system^[25], and this complex crosstalk is under the control of immune tolerance^[26]. Why tolerance is lost and an abnormal response to otherwise normal gut bacteria develops in IBD is still not entirely clear. However, the recent discovery that CD is genetically associated with mutations of the NOD2/CARD15 gene, whose product is a bacteria-recognizing cytoplasmic protein, points to defective mechanisms of bacterial sensing as the link between the gut flora and the altered immune response found in IBD^[27].

CELLULAR FACTORS

The most common type of reaction that the body mounts against external or internal offending agents is inflammation. The gut is particularly susceptible to inflammation as indicated by the fact that, and even under normal circumstances, there is a baseline degree of “physiological inflammation” in the mucosa. This is caused by a tightly controlled immune response directed at an enormous array of dietary and microbial antigens, and it is translated by the presence of an abundant number of leukocytes in the lamina propria^[28]. The ultimate goal of an effective inflammatory response is to eliminate the offending agent(s) and then disappear once the cause of inflammation has been eradicated. If inflammation persists and becomes chronic, it represents an inappropriate response that almost invariably leads to lingering injurious effects resulting in anatomical and functional abnormalities. Both CD and UC are typical chronic inflammatory processes of the gut which, by definition, are due to abnormalities of the intestinal immune system. Fortunately, major advances have occurred during the past three decades in our understanding of the cellular and molecular mechanisms mediating mucosal immunity and the alterations that lead to chronic gut inflammation^[29].

Adaptive immunity

Abnormalities of intestinal immunity in IBD began to be described several decades ago in regard to the main effector cells of adaptive immunity; e.g., T- and B-cells. Initially, it was discovered that the production of antibodies, particularly IgG antibodies, in the systemic as well as mucosal compartments was drastically increased and that the relative proportions of immunoglobulin classes and subclasses were altered as a consequence of chronic gut inflammation^[30-32]. In parallel with these studies, the possibility that some of these antibodies were true autoantibodies directed at self-components of the gut began to be explored. A series of studies suggested that IgG1 antibodies against a structural protein of colonocytes were selectively produced in UC, but not in CD, and could underlie the pathogenesis of this condition^[33]. Until now, however, definitive proof for the existence of classical, tissue injury-inducing autoantibodies in UC is still missing. With the recognition of T-cells as central effector cells and their soluble mediators as key modulators of immunity, the focus of immune investigation in IBD shifted to T helper (Th) cell subsets and the soluble mediators they

produce. A large number of cytokine abnormalities have been described, including pro-inflammatory and immunoregulatory molecules^[34]. In CD, intestinal CD4+ T cells produce large amounts of INF- γ and display marked overexpression of the Th1-cell-specific transcription factor, T-bet^[35], while mucosal macrophages produce large amounts of IL-12 and IL-18^[36,37]. Additionally, CD mucosal T-cells are resistant to apoptosis and cycle faster than control cells^[38,39]. In contrast, in UC nonclassical CD1d-restricted NK T-cells produce increased amounts of IL-13, and mucosal T-cells produce more IL-5, cycle slower and die more than control cells^[39-41]. Based on these observations, it is now generally accepted that the two main forms of IBD are associated with distinct immune profiles which are classified as a fairly typical Th1 response in CD and an atypical Th2 response in UC.

More recently, the study of adaptive immune abnormalities in IBD has been focusing on possible defects of immunoregulation. Different types of immunoregulatory cells exist, the best defined being CD4 + CD25 high T-cells, which are critically important in preventing autoimmunity and suppressing excessive immune reactivity^[42]. In IBD there is a contraction of this regulatory cell pool in the blood and only a moderate expansion in the inflamed intestine, suggesting the presence of insufficient regulation during active disease^[43].

Innate immunity

With the discovery of an association of a group of CD patients (those with small bowel and stricturing disease) with mutations of the NOD2/CARD15 gene, whose product is found in cells mediating innate immunity (primarily macrophages and dendritic cells) and recognizes the bacteria-derived component muramyl dipetide (MDP)^[44,45], a surge of interest in the role of innate immunity in IBD has occurred. Dendritic cells are scarce in the gut mucosa, but form a heterogeneous population of potent antigen-presenting cells pivotal to the balance between tolerance and active immunity and controlling the type of response - inflammatory or not - that follows detection of commensal bacteria^[46]. In IBD, mucosal dendritic cells are activated, express increased levels of the toll-like receptors (TLR) 2 and TLR4- which mediate recognition of bacterial products - and CD40, and produce more IL-12 and IL-6^[47]. All of these phenotypic and functional features indicate a prominent role of dendritic cells in IBD pathogenesis. Epithelial cells are also involved in innate immunity. Interestingly, ileal Paneth cells also express the NOD2 protein, and their production of mucosal α -defensins is decreased in CD patients with NOD2 mutations, perhaps leading to an impaired resistance against enteric microorganisms and eventually contributing to bacteria-induced inflammation^[48].

Another crucial component of innate immunity is the TLRs, cell surface molecules that detect microbial infection and trigger antimicrobial host defense responses^[49]. TLRs are abundantly expressed on the surface of monocytes, macrophages, and dendritic and epithelial cells and, in addition to recognizing pathogenic microorganisms, are essential to identify the commensal microflora and maintain intestinal homeostasis^[50]. Alterations of TLR3 and

TLR4 expression by intestinal epithelial cells have been described in IBD, suggesting the possibility that abnormal bacterial sensing contributes to disease pathogenesis^[51]. Because both NOD2 and TLRs are involved in innate immunity and recognition of and response to bacteria, much attention has been recently devoted to their biological interrelationship and the possibility of functional abnormalities in IBD, and CD in particular. Monocyte-derived macrophages of CD patients carrying homozygous mutations of NOD2 show clear-cut defects of IL-1 β and IL-8 production upon activation by MDP or TNF- α ^[52]. Moreover, the synergism between MDP and TLR ligands that causes a substantial upregulation of TNF- α and IL-1 β production in normal peripheral blood mononuclear cells is lost using cells from CD patients with double mutant genotypes^[53]. Thus, these preliminary reports point to the existence of generalized major defects of innate immune responses mediated *via* pattern recognition receptors in CD.

Nonimmune cells

Other cell types participate in the chronic inflammatory response of IBD, including epithelial, mesenchymal and endothelial cells, and platelets, which actually exert many of the functions traditionally attributed to classical immune cells, such as cytokine production or expression of MHC class II antigens.

Initial evidence that intestinal epithelial cell (IEC) function may be altered in IBD was acquired when immunohistochemical studies showed that IEC inappropriately expressed the class II antigens HLA-DR in actively inflamed mucosa of UC and CD patients^[54]. Later on, after the demonstration that normal IECs have antigen-presenting capacity and preferentially stimulate CD8+ suppressor T-cells, a report showed that IEC from IBD mucosa fail to induce such cells and instead activate CD4+ T-cells, and thus potentially amplify intestinal inflammation^[55]. More recently, IBD IECs were reported to inappropriately express members of the B7 family of co-stimulatory molecules^[56], a finding suggesting possible alterations in B7-ICOS costimulatory pathways in IBD. These reports, together with the above-mentioned altered expression of TLRs in IBD^[51], provide support for the notion that IECs have a role in IBD pathogenesis, but to fully understand their functional relevance will require additional investigation.

The involvement of fibroblasts in IBD has been traditionally viewed as one restricted to production in the extracellular matrix and the pathogenesis of a common and serious complication; e.g., intestinal fibrosis^[57,58]. However, fibroblasts are also involved in gut injury because they represent a major source of matrix metalloproteinases (MMPs), a family of proteolytic enzymes directly responsible for tissue destruction during inflammation^[59,60]. Of special importance is the observation that interaction with activated T-cells is a major pathway of fibroblast activation and MMP production, a phenomenon that links together fibroblast function, adaptive immunity, and gut tissue injury^[61]. In reality, the functional interaction of mucosal fibroblasts with the surrounding microenvironment is physically more complex and functionally more important than previously recognized. A

recent report showed that activation of fibroblasts through the CD40 pathway induces the upregulation of cell adhesion molecules and production of chemokines which, in turn, induce the migration of T-cells through local microvascular cells^[62]. Therefore, mucosal fibroblasts must also be considered as active rather than passive participants in IBD pathogenesis.

Endothelial cells play an essential role in inflammation due to their central "gatekeeper" function, which controls the quality and quantity of leukocytes that transmigrate from the vascular into the interstitial space. This process is complex and is mediated by a number of molecules, including cytokines, chemokines and adhesion molecules. A key observation that opens the whole field of functional vascular biology in IBD is that human intestinal microvascular endothelial cells (HIMEC) isolated from CD and UC mucosa exhibit a significantly higher cytokine-mediated leukocyte binding capacity compared to HIMEC from normal mucosa^[63], a phenomenon secondary to their chronic exposure to the inflammatory milieu of the IBD mucosa^[64]. Increased leukocyte adhesion by IBD HIMEC is apparently due to their deficient production of inducible nitric oxide (NO) synthase^[65]. This also causes a microvascular endothelial dysfunction in IBD due to a loss of NO-dependent dilation that may lead to reduced perfusion, poor wound healing, and maintenance of inflammation^[66].

The role of platelets in IBD has been known for quite some time, but primarily because of their involvement in thrombotic events which are relatively common in CD and UC patients^[67]. However, platelets have increasingly acquired a strong immunological connotation through the demonstration of their initiator or amplificatory role in immunity and inflammation, which is mostly mediated through the CD40/CD40 ligand pathway^[68]. Platelets exist in an activated state in the peripheral circulation of IBD patients, and the elevated levels of soluble CD40 ligand present in their systemic circulation are mostly of platelet origin, apparently due to platelet activation in the inflamed intestinal microvascular bed^[69]. More importantly, recent studies have shown that platelets trigger a CD40-dependent inflammatory response in the microvasculature of IBD patients^[70], thus closely linking this unique cell type to the process of IBD pathogenesis.

CONCLUSIONS

Since the recognition of IBD as a perplexing and challenging clinical entity, the investigation of its pathogenic mechanisms has gone through repeated cycles of new hopes, new knowledge, and new realities. Infectious, allergic, dietary, psychosocial, environmental, microbial, vascular, metabolic, immune and other based theories have been put forward, most of them to be rebuked, if not ridiculed^[71]. At the moment, we appear to have settled down on a unifying but still wide-ranging hypothesis that IBD results from complex interactions between evolving environmental changes induced by society's progress, a still undefined number of predisposing genetic mutations, an incredibly complex gut microbiota that may be constantly varying, and the intricacies of

individual immune systems^[72]. The ability to integrate all these various components into a single cohesive and logical pathway of disease that explains all aspects of IBD appears still a bit distant at the moment. On the other hand, if we look back at where we stood only two or three decades ago, the progress achieved in our understanding of IBD pathogenesis and the way it has changed our approach to therapy is just short of spectacular.

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Paolo Gionchetti, MD, Series Editor

Cytomegalovirus and inflammatory bowel disease: Is there a link?

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Abstract

The objective of this report is to give an overall view of the epidemiological, clinical, diagnostic and therapeutic features of Cytomegalovirus (CMV) infection in inflammatory bowel disease (IBD). A review of published reports on this topic was carried out, with particular attention paid to the selection of patients included in studies and the diagnostic methods employed. CMV is frequently associated with IBD. In some cases, CMV infection is associated with a poor outcome but it is not clear which patients are more likely to be affected and in which stage of the disease. The use of anti-viral therapy in IBD is controversial and an empirical study with controls is needed. The natural history of CMV infection related to the development and treatment of IBD has not been clarified but it is important to take it in consideration because of the possibility of viral persistence in the immunocompromised host and viral interaction with the immune system.

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Key words: Cytomegalovirus; Inflammatory bowel disease; Outcome

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INTRODUCTION

Cytomegalovirus (CMV) is a member of the Herpesviridae

family, which includes Epstein -Barr virus (EBV), Herpes Simplex virus types 1 and 2 (HSV-1,2), Varicella- Zoster virus, and Human Herpes virus types 6 and 7 (HHV-6,7). Similar to infection with other viruses in the family, primary infection with CMV results in the establishment of a persistent or latent infection, due to the ability of the virus to remain integrated in the DNA of host cells. The sign of a viral infection is a cytopathic effect shown by the presence of large nuclear and cytoplasmic inclusions, represented by aggregates of replicating CMV nucleoprotein cores. To avoid recognition and destruction by CD8+T lymphocytes, the virus develops the ability to evade the immune system by several mechanisms. CMV produces some proteins, such as US2, US3 and US11, that inhibit the presentation of viral antigens to T cells, blocking the class I MHC in the endoplasmic reticulum or in the cytosol. It also produces homologues to class I MHC proteins and may compete for binding and presentation of viral antigens. Once the viral DNA remains undisturbed in the infected cells, subsequent reactivation can occur in response to several stimuli, such as immunosuppressant therapy or chemotherapy^[1]. The down-regulation of the cell surface markers acts on interferon-alpha/beta dependent responses by affecting several levels of IFN signal transduction and a transcription activation pathway. CMV infection leads to the activation of Nuclear Factor kappa B (NF-κB) and its translocation to the nucleus, promoting the expression of cytokines, chemokines and cellular adhesion molecules. These mechanisms are also present in inflammatory bowel disease (IBD) where there is activation of several pro-inflammatory cytokines (such as IFN-gamma), the transcription of which is regulated through nuclear transcription factors (such as Nuclear Factor kappa B) and through a signal transducer and activator of transcription (STAT family).

CMV AND IBD

The role of CMV in IBD is reviewed considering the following issues: diagnostic methods to detect the virus, prevalence of CMV in IBD according to patient selection, clinical characteristics, outcome for patients with IBD and superimposed CMV infection, role of antiviral therapy and natural history of CMV in IBD patients.

Diagnostic method

Results differ based on diagnostic method. Serology is

useful in checking for previous virus exposure and for identifying patients at risk. Since gross appearance is non-specific, a diagnosis is based on the histopathological identification of viral-infected cells in biopsied tissues, using appropriate staining Haematoxylin and Eosin (HE), Immunohistochemistry (ICH), by a dedicated pathologist. Cytomegalic cells have been revealed that are 2- or 4 fold larger (25-35 μm) than surrounding cells, containing a basophilic intranuclear inclusion (8-10 μm) that is eccentrically placed and is sometimes surrounded by a clear halo, giving it an "owl's eye" appearance. These cells also show a thickened nuclear membrane, frequently associated with smaller granular intracytoplasmic inclusions.

Intranuclear inclusions were observed in epithelial, endothelial, stromal and smooth muscle cells.

In the gastrointestinal tract, "atypical" inclusions were also found^[2]. Biopsies taken from the mucosa near or within the ulcer provided greater detection of the virus. IHC is more sensitive than HE.

The CMV pp65-antigenemia assay as Polymerase Chain Reaction (PCR) DNA amplification is a sensitive, specific and rapid method for the early diagnosis of CMV infection based on immunocytochemical detection of a virus protein (pp65) in the nuclei of peripheral blood polymorphonuclear leukocytes. The advantage of this method is an early and rapid response possibility but this technique does not permit the distinction between an asymptomatic infection and an active disease. Viral load quantification may permit the observation of the infection course. PCR is a very sensitive test for the detection of CMV. In solid organ transplant patients, CMV detection can persist for months using qualitative PCR assays, despite effective antiviral therapy. Therefore, the precise amount of CMV DNA may be better determined with virological monitoring. In addition to studies using quantitative PCR in renal transplant recipients, studies on peripheral blood leukocytes suggest a cut-off of > 1000 copies/100 000 leukocytes, indicative of the development of symptomatic CMV infection after transplant^[3]. A slow or absent decline in CMV DNA after the beginning of ganciclovir therapy could be an early indicator of drug resistance^[4]. A PCR positive assay after ganciclovir therapy, irrespective of resolution of clinical signs and symptoms, might be an indicator that therapy should be continued^[5]. Mendez *et al*^[6] analyzed the early diagnosis of CMV infection using four sets of primers that were able to amplify different regions of the CMV genome. The authors demonstrated that a specific primer directed to the HIND III-X fragment region is the most sensitive primer for the early detection of CMV DNA in peripheral blood leukocytes (PBLs), providing detection 17 d before the onset of the symptoms. *In situ* hybridization and qualitative PCR in colonic biopsies seem to offer the greatest accuracy^[7-9] in detecting the virus.

Quantitative PCR (viral-load) in cells from the colon^[10] showed a high positive-predictive value for detecting disease and for monitoring therapeutic response. A qualitative and quantitative PCR assay for CMV DNA has been performed on human faecal specimens from immunocompromised patients^[11,12].

Patient selection method

The reports on prevalence of CMV are extremely varied in regard to patient selection methodology. There is no study that gives an overall prevalence of CMV in IBD patients. Most of the studies have been carried out using a selected patient group (severe colitis, steroid-resistant colitis, urgent colectomy for colitis, patients with active disease) and different diagnostic methods were used as well for different patient group (e.g., histology, ICH, antigenemia, electron microscopy, *in situ* hybridization). Prevalence has been reported to range from 0.5 to 100% (Table 1).

Severe colitis: A group in Italy^[13] studied the prevalence of CMV using rectal biopsies and HE staining, immunoperoxidase staining for CMV antigens and antigenemia pp65 (a buffy-coat preparation) on the peripheral leukocytes. The study was performed prospectively in a consecutive series of hospital admissions without waiting for a possible response to conventional therapy, in order to determine the prevalence of CMV in severe consecutive episodes of colitis. The results showed virus prevalence overall in patients with active IBD (21%). On the basis of these results the authors suggested performing a flexible proctoscopy (without air insufflation) with rectal biopsies in patients hospitalized for severe colitis flare-up, together with antigenemia on peripheral leukocytes, to determine whether the simultaneous detection of virus both in the colon mucosa and in the peripheral blood may be interpreted as the pathogenic cause.

Khishore^[14] used serology, HE and PCR for CMV DNA to study a heterogeneous population, including patients with severe colitis. Thirty-six patients had severe colitis, with 8 patients (22%) shown to be CMV positive based on colonic biopsies. Moreover, the author identified clinical variables associated with a higher risk of CMV infection in IBD. These factors included female gender, pancolonic disease with active inflammation at histology, and azathioprine treatment. Using antigenemia, Wada^[15] reported a prevalence of 34%, while Vega^[16] reported a result of 3% in a retrospective study that used HE and ICH results.

Severe steroid-resistant colitis: Cottone *et al*^[17] showed that the prevalence of CMV, studied using HE and ICH and antigenemia, was 36%. Kambham^[18] obtained a similar result, using HE and ICH on colonoscopic biopsy specimens, detecting CMV in 4 of 15 steroid-resistant patients (26%). Pofelsky^[19], using PCR, showed a higher prevalence (60%) compared to previous reports, and an overall prevalence of viral DNA in the colon of 38%. However, there was a poor correlation between colonic and peripheral viral load which suggests a role of local inflammation in the colon. The genotype detected was gB1, which possibly has a particular colonic tropism. On the contrary, Papadakis^[20], in a retrospective study on 1895 patients, showed a low rate of 0.5% prevalence. He suggested a pathogen role for the virus based on the prompt response and clinical improvement found with antiviral treatment.

Urgent colectomy for colitis: These patients represent a subgroup in which IBD is more severe and, therefore, a higher prevalence of CMV is expected. Six studies

Table 1 Prevalence data according to population and methodology of the study

Author (year of publication)	Population characteristics	Diagnostic assay	Study type	Prevalence
Criscuoli (2004)	42 pts with severe colitis	HE-ICH on rectal biopsy + antigenemia pp65	prospective	21%
Kishore (2004)	63 pts with severe colitis (36 steroid resistant)	Serology-HE and qualitative PCR	prospective	16% 22%
Wada (2003)	47 pts with severe colitis	Antigenemia	prospective	34%
de Saussure (2004)	64 pts with active colitis	Serology-viremia-antigenemia- HE-ICH	prospective	1.5%-6%
Vega (1999)	267 active colitis	HE-ICH	retrospective	3%
Pofelski (2005)	48 pts severe colitis	Quantitative PCR on colonic biopsies	retrospective/ prospective	38% 60%
Kambham (2004)	40 pts steroid resistant (25 operated on for colectomy)	HE-ICH	retrospective	Overall 25% Operated on pts 24%
Papadakis (2001)	1895 steroid resistant pts	HE	retrospective	0.5%
Cottone (2001)	19 Steroid resistant pts	HE-ICH on rectal biopsy + antigenemia pp65	prospective	36%
Maconi (2005)	77 pts operated on for colectomy	HE-ICH on surgical specimen	prospective	22% overall 27% steroid resistant
Takahashi (2004)	69 surgical specimen of IBD pts	HE-ICH on biopsy and surgical specimen	retrospective	11.5%
Alcalà (2000)	39 pts operated on for colectomy	HE + ICH	retrospective	18%
Eire-Brook (1986)	26 pts operated on for colectomy	Light and electron microscopy- ICH	retrospective	11.5%
Cooper (1977)	46 pts operated on for colectomy	HE	retrospective	13%
Rahbar (2003)	23 pts with IBD (13 UC-10 CD)	ICH+ <i>in situ</i> hybridization	prospective	92/100%
Wakefield (1992)	50 pts with IBD (29 CD-21 UC)	qualitative PCR	prospective	66/81%

(two older studies using HE and four recent studies using ICH) have been reported on surgical specimens from patients with colitis who were not responsive to medical therapy^[6,21-25]. These studies showed an overall prevalence (11.5%-27%) that is similar to reports in other studies. In the subgroup of steroid-resistant patients, Kambham and Maconi reported a similar prevalence of 25%-27%.

Patients with active disease: Rahbar^[7] has estimated virus prevalence in intestinal biopsies from IBD patients both using ICH and *in situ* hybridisation. The latter showed detection in over 90% of ulcerative colitis (UC) patients, and in 100% of patients with Crohn's disease (CD). Since the presence of the virus does not necessarily mean active infection, the authors looked at viral replication by CMVea (early antigen) and CMVla (late antigen) by immunohistochemistry, and they obtained a similar result (85% *vs* 100% for UC *vs* CD, respectively) using CMVea.

Other authors have used an immunoperoxidase technique, using a monoclonal antibody against CMV, to demonstrate early CMV infection in cells of colonic specimens in cases showing few cytopathic cells at histological examination. On the contrary, the DNA *in situ* hybridization technique was less helpful in establishing a diagnosis of early infection^[8].

A study that used PCR showed positive detection in about 66% of CD patients, 81% in UC patients, and 29% in controls^[9].

CMV and acquired immunodeficiency syndromes or immunosuppressive therapy

The gastrointestinal system is a common site of CMV infection, especially in AIDS patients. Any tract can be affected, with preference to oesophagus and colonic mucosa (especially right colon) rather than ileum, considering the pouch mucosa are morphologically similar

to colon mucosa^[26]. The syndrome begins with a watery diarrhoea due to an inflammatory response, that quickly turns into bloody diarrhoea due to ulcerative changes in the colonic mucosa. The endothelial cells are a site of CMV detection both in a latent state and active replication state^[27]. This explains why CMV vasculitis is a common manifestation of viral disease localized in different organs (bowel, retina, brain) and that vascular damage is responsible for thrombosis^[28] and atherosclerosis. In the bowel, vasculitis may cause ischemia and transmural necrosis with an increased risk of toxic megacolon and perforation.

A recent meta-analysis^[29] on the outcome of CMV colitis in an immunocompetent host reviewed 44 cases (of which 16 had coexisting immune-modulating morbidities such as diabetes, malignancy or renal failure). The conclusion of this analysis was that CMV colitis is found more frequently in elderly patients in whom the disease had a severe course and where there was a high mortality rate. On the contrary, younger patients (< 55 years) had a significant rate of spontaneous recovery, with 42% of patients diagnosed with subsequent IBD after resolution of CMV infection. Ng^[30] carried out a retrospective analysis on patients without apparent causes for immunodeficiency but who were mainly elderly and were admitted to hospital with bloody diarrhoea. The mortality rate reported was 40% which was thought to be related to co-morbidity

IBD and superimposed CMV infection: When the syndrome appears with a pre-existing inflammatory disease, IBD may be more aggressive. The clinical outcome of IBD with superimposed CMV is not well understood. The first report about the possible role of cytomegalovirus in IBD dates to 1961 when Powell^[31] described a case of ulcerative colitis and cytomegalic inclusion disease. After many sporadic reports over the last decade, the topic has

regained attention due to more frequent publications of case-reports or small series studies in which the virus provided a worsening prognosis influence, sometimes promoting disease initiation or otherwise acting as a bystander^[9].

The coincidental detection of primary CMV infection at the first appearance of IBD is reported in some case-reports^[32,33], underlining the ability of CMV proteins to enhance pro-inflammatory cytokines that are able to maintain a local colonic inflammation with an immune response. In other conditions the latent virus could exacerbate^[34-36] pre-existing colitis after immunosuppressive situations. Experimental studies have shown that highly proliferating cells, like those in the granulation tissue, around inflammation or in ulcer depth, are easily objects of CMV infection. In this situation the virus could reach the mucosa by monocytes and then colonize the mucosa, acquiring particular affinity for the inflamed mucosa.

This is evidenced by the fact that super-infection with pre-existing colitis causes worsening of symptoms, with a severe course of disease that rarely strikes suddenly, with high prevalence of toxic megacolon and surgical intervention^[10,11,33]. In this case, since steroids or immunosuppressive therapy can lead to the flare-up of CMV infection, the outcome for patients with an acute attack is likely to be poor. A case of disseminated (whole gastrointestinal tract, skin and central nervous system) CMV infection has been reported in Crohn's disease after anti-TNF therapy^[37]. The mechanism of dissemination is likely to be related to vascular damage that allows a viral circulation within the shed endothelial cells.

The literature contains many case reports of CMV infection in steroid-naïve patients or immunocompetent hosts with IBD. Rachima^[38] reported two cases of CMV infection diagnosed by high titres of IgM antibodies to CMV (solid-phase enzyme immunoassay) but without histological detection on colonic biopsies. A high prevalence of CMV IgG antibody in patients with ulcerative colitis, compared to normal controls and to patients with active Crohn's disease, permits the hypothesis of a possible role for the virus to exacerbate the inflammatory disease and therefore its pathogenicity. Although clinically significant CMV infection occurs in individuals with acquired immunodeficiency syndrome or due to immunosuppressive therapy, the same inflammatory disease could be considered a booster of viral infection with local factors, even without previous immunosuppressive therapy.

Antiviral treatment: A review of the literature does not affirm that antiviral treatment is mandatory when CMV is detected in biopsy specimens or in peripheral blood, however, some authors are favourable to the use of antiviral treatment^[2-9]. Eddleston^[39] proposes the consideration of antiviral therapy in immunocompetent patients with multiple organ disease, taking into consideration the poor prognosis with widespread CMV disease. Pfau considers Ganciclovir as a beneficial treatment that significantly decreases the mortality rate and the request for surgery. Eire-Brook considers CMV as a bystander and refuses to allow any antiviral treatment during an acute flare-up of IBD.

It is difficult to draw conclusions about the role of antiviral therapy on the basis of the available evidence. Se-

vere steroid-resistant colitis is the setting in which the role of antiviral therapy should mainly be considered. According to some reports, antiviral therapy treatment was useful, whereas in others it was useless. A controlled study is necessary to answer this question, however a large number of patients would be needed for such a trial and it would therefore be necessary to involve many centres using homogeneous diagnostic methods.

Natural history of CMV infection: No studies have reported on the natural history of CMV infection. It is not known whether the virus remains in the colon after an acute attack or spontaneously disappears. It is also not known whether there is a high incidence of relapse when the virus remains in the colon. Furthermore, it is not clear whether the virus plays a role in risk for cancer and lymphoma of IBD. For many years, the involvement of Human Herpes Virus (EBV, HHV-8) has been well known in the pathogenesis of some tumours, while there is little knowledge about the potential oncogenetic role of CMV.

A study performed using ICH, *in situ* hybridisation and PCR on pathological specimens of colorectal polyps, adeno-carcinomas and normal mucosa, reported an immunoreactivity of at least 80% in the colorectal polyps and carcinoma both for an immediate early gene product (IE1-72) and for a delayed early gene product (pp65), and acid nucleics. Moreover, detected cyclo-oxygenase -2 in the same site of virus detection may play an important oncogenetic role in the development of human colorectal cancer. The virus could activate cellular proto-oncogenes, kinases and transcription factors implicated in tumour-cell survival pathways^[40].

On the contrary, a more recent study^[41] using similar methods did not find nuclear immunoreactivity for CMV proteins and DNA, but found focal cytoplasmic positivity in normal colonic mucosa as well, demonstrating no evidence of association. Adani^[42] reported a case of severe ulcerative colitis with superimposed colonic CMV infection without peripheral involvement that was associated with colorectal high-grade B-cell non Hodgkin's lymphoma (MALT type). More frequent association is reported between CMV pneumonitis and non-Hodgkin's lymphoma. Both cases were associated with previous immunosuppressive treatment, which is a well-known promoting factor.

CONCLUSION

In general the role of CMV in IBD remains unclear. On the basis of the current evidence, in our opinion, in active severe IBD CMV, if is detected in the colonic biopsies together at the presence of antigenemia, should be treated with antiviral drugs. There is certainly a pathogenic role of CMV in immunosuppressed transplant patients and in patients with AIDS where treatment is mandatory.

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Extraintestinal manifestations and complications in inflammatory bowel diseases

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Abstract

Crohn's disease (CD) and ulcerative colitis (UC) are chronic inflammatory bowel diseases (IBD) that often involve organs other than those of the gastrointestinal tract. These nonintestinal affections are termed extraintestinal symptoms. Differentiating the true extraintestinal manifestations of inflammatory bowel diseases from secondary extraintestinal complications, caused by malnutrition, chronic inflammation or side effects of therapy, may be difficult. This review concentrates on frequency, clinical presentation and therapeutic implications of extraintestinal symptoms in inflammatory bowel diseases. If possible, extraintestinal manifestations are differentiated from extraintestinal complications. Special attention is given to the more recently described sites of involvement; i.e. thromboembolic events, osteoporosis, pulmonary involvement and affection of the central nervous system.

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Key words: Inflammatory bowel diseases; Crohn's disease; Ulcerative colitis; Extraintestinal manifestations; Complications; Therapy

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INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC) are the main entities of chronic inflammatory bowel diseases (IBD). Although in most cases the gastrointestinal tract is

mainly affected, both ulcerative colitis and Crohn's disease are systemic disorders that often involve other organs. These nonintestinal affections are termed extraintestinal symptoms and may not always coincide with the underlying bowel disease. Extraintestinal disease can involve almost every organ system. The organs most commonly involved include the skin, eyes, joints, biliary tract and lungs. Some symptoms, such as oral lesions, gallstones, pancreatitis, nephrolithiasis and amyloidosis, are more associated with CD than with UC. Other symptoms, e.g. skin and eye manifestations, are equally seen in both CD and UC.

Several factors may be responsible for extraintestinal organ involvement in IBD and sometimes it can be difficult to differentiate the true extraintestinal manifestations (EIMs); i.e. primary systemic affection by the disease itself, from secondary extraintestinal complications of the disease, caused for example by malnutrition, chronic inflammation or side effects of therapy. Some of these EIMs may not correlate with disease activity (primary sclerosing cholangitis and ankylosing spondylitis) but in general EIMs tend to follow the clinical course of IBD and may have a high impact on quality of life, morbidity and even mortality in these patients.

The reported frequency of EIMs in patients with IBD varies from 6%-47%^[1-4]. The development of one EIM appears to increase the susceptibility of developing other EIMs. An overlap of EIMs is particularly observed with peripheral arthritis, erythema nodosum, affection of the biliary tract and the eyes, in concordance with the hypothesis of a common pathogenic pathway. Some authors discuss an autoimmune reaction towards an isoform of tropomyosin (Tropomyosin related peptide), which is expressed in eye (non-pigmented ciliary epithelium), skin (keratinocytes), joints (chondrocytes), biliary epithelium and the gut^[5,6].

The high concordance in EIMs in siblings and first degree relatives with IBD^[7] suggests a common genetic background. Crohn's disease and ulcerative colitis are polygenic disorders and certain susceptibility genes in the major histocompatibility complex (MHC) region on chromosome 6 seem to be linked to EIMs in IBD. In CD, extraintestinal co-morbidities are more commonly observed in patients with HLA-A2, -DR1 and DQw5, whereas in ulcerative colitis, the genotypes HLA-DRB1*0103, B*27 and B*58 are linked with EIMs involving the joints, skin and eyes^[8-10]. Fifty to eighty percent of IBD patients with ankylosing spondylitis are

HLA*B27 positive^[11]. Furthermore, in UC the haplotype HLA B8/DR3 is associated with primary sclerosing cholangitis (PSC) and may also be linked to other autoimmune diseases (e.g. celiac disease, autoimmune hepatitis, myasthenia gravis)^[12]. Interestingly, the NOD2 gene in CD seems to be associated not only with ileal disease in CD but also with sacroileitis^[13].

In the following, an overview of the involvement of the different organ systems in IBD will concentrate on frequency, clinical presentation and therapeutic implications. In some cases, a differentiation of extraintestinal *manifestations* and extraintestinal *complications* will be given. Besides the classical extraintestinal manifestations, such as skin, joints, eyes and the hepatobiliary system, special attention will be given to the rarer, more recently described involvements, such as thromboembolic events, osteopenia and osteoporosis, pulmonary involvement and affection of the central nervous system.

MUSCULOSKELETAL MANIFESTATIONS

Joint manifestations are the most common EIMs in IBD and occur in approximately 20%-30% of patients^[3,14]. Males and females are equally affected. Symptoms may range from arthralgia only to acute arthritis with painful swollen joints. Both peripheral arthritis and axial arthritis can occur.

Peripheral arthritis

Peripheral arthritis in IBD is quite distinct from specific forms of arthritis since there is little or no joint destruction, and tests for rheumatoid factor, antinuclear antibody and LE factor are negative. The prevalence of all forms of peripheral arthritis is reported to be between 5%-10% in UC and 10%-20% in CD, respectively^[6,15], not considering asymptomatic patients under medical treatment. There are two types of peripheral arthritis in IBD^[15] that should be distinguished from unspecific myalgia or arthralgia:

Type 1 (pauciarticular) arthritis affects less than five large joints (predominantly of the lower limbs) and the swelling is acute and often self-limiting. Type I arthritis is related to disease activity of the underlying bowel disease. The mean duration is 5 weeks; some 25%-40% of patients will have recurring arthritis. Type 2 (polyarticular) arthritis is a symmetrical polyarthritis, frequently involving five or more of the small joints (e.g. knuckle joints). Its course is independent of disease activity and may last for several months.

The etiology of peripheral arthritis in IBD is thought to be a combination of genetic predisposition and exposition to luminal (bacterial) bowel contents. Type 1 IBD arthritis is associated with HLA-DRB1*0103, HLA-B*27 and HLA-B*35, whereas type 2 IBD arthritis is associated with HLA-B*44 and MHC class I chain-like gene A, which is a non classical HLA gene located near the HLA-B on chromosome 6^[8,10,16]. The site of intestinal inflammation is of particular interest concerning the pathogenesis of joint inflammation since CD patients with colonic involvement are at higher risk of developing arthritis than those with

isolated small bowel disease. Furthermore the incidence of new joint complications significantly decreases after ileocecal resection (even when corrected for the time spent in remission after surgery), suggesting that bacterial overgrowth proximal to the ileocecal valve plays an important role in the pathogenesis of extraintestinal joint inflammation^[6].

The diagnosis of peripheral arthritis in IBD is made clinically since radiographic findings do not show erosions or deformities. In persisting disease, a positive rheumatic factor should be excluded. In acute swelling, septic arthritis, fistulating arthritis or gout may be excluded by joint aspiration.

Treatment: Type 1 arthritis is related to disease activity and therefore therapy of the underlying IBD is the treatment of choice. Especially in patients with relapsing arthritis (HLA-DRB*0103), 5-ASA treatment should be switched to sulfasalazine, thereby taking advantage of the antiarthritic effect of sulfapyridine to minimize the risk of relapse. In addition, symptomatic treatment is often sufficient. For analgetic therapy, NSAID's and COX-2 selective inhibitors should be avoided, if possible, due to their potential to activate the underlying IBD^[17]. In severe cases, symptoms relief can be achieved by intra-articular steroid injection. Type 2 IBD arthritis generally requires long-term treatment. In persisting disease, sulfasalazine should be initiated at an initial dose of 2 × 500 mg per day, increasing the daily dose by 1000 mg every two weeks towards the maximum dose of 3 × 1500 mg or until symptoms improve. If not effective despite 12 wk of continuing treatment, immunosuppression with methotrexate (7.5 mg po once weekly) should be started. The dose can be increased by 2.5 mg steps in monthly intervals up to the maximum dose of 25 mg per week. Concomitant folic acid (5 mg po 24 h following methotrexate) is recommended to reduce side effects. Systemic corticosteroids may be necessary to control symptoms.

Axial arthropathies

The axial arthropathies are not associated with disease activity of IBD. Spondylitis occurs in 1%-26% of patients with IBD and males are more often affected than females. Both progressive ankylosing spondylitis and sacroiliitis (sometimes asymptomatic) may occur. Plain radiographs of the sacroiliac joints show uni- or bilateral sclerosis and/or erosions. The diagnostic gold standard is magnetic resonance imaging (MRI) with a high sensitivity in detecting sacroiliitis even in the absence of symptoms.

Ankylosing spondylitis

Ankylosing spondylitis (AS) affects the vertebral column by progressive ankylosis of the vertebral facet joints and the sacroiliac joint (Figure 1). The prevalence of AS in IBD (1%-6%) is higher than in the general population (0.25%-1%)^[18]. In contrast, the association with HLA*B27 is considerably weaker than in idiopathic AS with only 50%-80% of IBD patients being positive for HLA B*27 compared to 94% in the general population^[11]. Again, bacteria and gut inflammation seem to play an important role in the pathogenesis of AS.

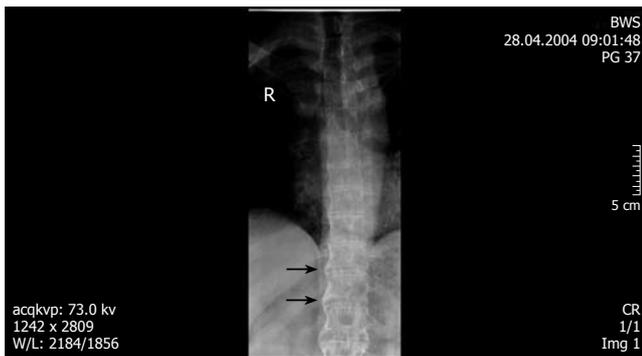


Figure 1 X-ray thoracic spine demonstrating ankylosing spondylitis (Bechterew's disease) with syndesmophytes/bamboo spine in a patient with CD.

Interestingly, ileocolonoscopy in patients with idiopathic spondylarthropathies reveals ileal inflammation in more than two thirds of patients^[19].

The clinical course of AS in IBD is similar to idiopathic AS, and disease progression leads to increasing immobility of the spine resulting in ankylosis (bamboo spine). Secondary to reduced chest expansion, poor lung expansion with fibrosis and dilatation of the aortic root can occur. AS is associated with peripheral arthritis in about 30% of patients and with uveitis in 25% of patients.

Treatment: There is no causative treatment and therefore physical therapy is of particular importance to maintain mobility of the spine. In the absence of active IBD, NSAID are the drugs of choice, otherwise acetaminophen or tramadol are preferred. Steroid injection (MRI-guided) into the sacroiliac joint may be an option in patients with severe low back pain^[20]. Sulfasalazine may be used, but is more effective in associated peripheral arthritis. The first line immunosuppressant in patients with AS and IBD is methotrexate. Anti-TNF-strategies should be reserved for severe cases. Experience is limited to small case series, but improvement of both spondylarthropathy and active bowel disease has been reported in CD with infliximab^[21-23]. Etanercept is effective in spondylarthropathies^[24,25] but the efficacy in CD has not yet been demonstrated.

Isolated sacroiliitis

It may occur in patients with IBD but most patients are asymptomatic and the disease is non-progressive. Prevalence depends on the radiological method used and varies from 18% in plain radiographs and 32% in CT imaging to 52% in radioisotope scintigraphy. Isolated sacroiliitis seems not to be associated with HLA*B27^[6]. Asymptomatic HLA*B27 negative patients with normal spinal mobility do not require specific treatment.

Osteoporosis

Patients with IBD have an increased risk of developing osteoporosis, associated with fragility fractures and morbidity. The overall prevalence of osteoporosis in IBD is approximately 15% but is more prevalent with older age; the overall relative risk of fractures is 40% greater when compared to the general population^[26,27]. Vertebral fractures often occur spontaneously or after minimal

trauma and it is estimated that only one-third of vertebral fractures come to clinical attention^[28]. X-ray images of the spine most commonly show wedge or compression deformities. A variety of studies have demonstrated both decreased bone mineral density (BMD) in patients with IBD^[29-38], and increased rates of bone loss when followed longitudinally^[34,39,40] in comparison to healthy controls. The current Gold standard for measuring bone mass is dual-energy X-ray absorptiometry (DEXA)^[41].

The pathogenesis of osteoporosis in IBD is multifactorial. Important pathogenetic factors in IBD include the cumulative steroid dose, hypogonadism induced by IBD (absence of menstrual period in women), malabsorption of calcium and vitamin D, low body mass index and disease activity/elevated inflammatory cytokines^[42]. Other risk factors are previous fragility fracture, a positive family history, concomitant liver/endocrine disease (hyperthyroidism, hyperparathyroidism), immobilization and life style risk factors (smoking, excessive alcohol intake, physical inactivity). The multifactorial pathogenesis of bone loss in IBD makes it difficult to assess the importance of each single contributing factor. The results of a study from Norway indicate that disease activity and corticosteroid therapy are the most important factors involved in bone loss in CD patients^[43]. However, it remains unclear whether the bone loss is related to the disease or to its treatment.

Biochemical markers of bone turnover (e.g., osteocalcin, bone specific alkaline phosphatase, carboxyterminal propeptide procollagen type 1, urinary deoxypyridinoline, pyridinoline, carboxytelepeptide of type I collagen, N-telopeptide cross-linked type I collagen) do not correlate sufficiently with current BMD for routine use^[30,33,35,44-46] and should be confined to research studies^[26].

Treatment: Few IBD patients are receiving optimal bone-sparing therapy, highlighting the importance of increasing awareness of osteoporosis in managing these patients^[38]. Preventing bone loss should begin with an attempt to limit corticosteroid-induced bone loss. This can be done by minimizing the corticosteroid dosage, substituting with budesonide when appropriate^[47,48], administering other steroid-sparing immunomodulators once corticosteroid dependence becomes evident, or by prescribing additional agents that enhance bone health. The administration of calcium and vitamin D^[49] appears to maintain or enhance bone mass^[26]. Bisphosphonates are of unclear additional benefit to the majority of patients who are at low fracture risk. In a small trial in Denmark, one year of daily alendronate treatment p.o. improved BMD in the spine^[50]. Bisphosphonates (etidronate, risedronate, alendronate) are effective in preventing bone loss in steroid treated patients, but only few patients with IBD have been included in these trials^[51-54]. Nasal or s.c. calcitonin can be considered as an alternative treatment approach when bisphosphonates are contraindicated or poorly tolerated. Testosterone replacement should be considered in hypogonadal men^[26], estrogen replacement in postmenopausal women^[55].

Osteomalacia

Osteomalacia is a rare complication of IBD, most likely occurring in patients with severe CD and multiple

intestinal resections^[56], resulting from prolonged and severe vitamin D deficiency. Though both osteoporosis and osteomalacia result in low BMD, apart from elevated bone alkaline phosphatase levels, osteomalacia can only be distinguished from osteoporosis by bone biopsy.

Joint complications in IBD

Complications involving the joints should always be considered and have to be distinguished from sterile joint inflammation, since steroid treatment can cause osteonecrosis (avascular necrosis of the bone). Patients on immunosuppressive therapy are at increased risk of septic arthritis. In CD, fistulization may cause bacterial infection of the iliosacral joint. Rarely a psoas abscess can cause septic hip arthritis.

MUCOCUTANEOUS MANIFESTATIONS

Erythema nodosum, pyoderma gangraenosum and oral ulceration are the most common cutaneous manifestations in IBD and are usually related to disease activity but sometimes may take an independent course. All patients presenting with IBD should be examined for cutaneous manifestations.

Erythema nodosum

It is the most common skin manifestation in IBD affecting up to 15% of CD patients, with a female predominance^[1,6,14]. Erythema nodosum (EN) affects the subcutaneous fat (septal panniculitis), causing tender erythematous nodules usually located on the shins (Figure 2). EN normally heals without ulceration and the prognosis is good. The clinical picture is typical and biopsy rarely is required for diagnosis. The etiology of EN is unknown, however there is a genetic association with a distinct HLA region on chromosome 6 (HLA-B^[9]). EN characteristically parallels intestinal disease activity. There is an association with other EIMs such as arthritis and uveitis^[14,15]. Treatment of the underlying bowel disease usually results in improving EN lesions and at least 25% of EN will heal spontaneously. There is no specific treatment for EN, but symptomatic therapy should comprise pain medication and oral steroids may be given in severe cases. Immunosuppressive treatment is not necessary. In the absence of active bowel disease, other causes of EN, such as sarcoidosis or post-streptococcal infection, should be taken into consideration.

Pyoderma gangrenosum (PG)

Pyoderma gangrenosum occurs in 0.5%-2% of both patients with UC and CD and may take a course independent of disease activity (Figure 3). Conversely, 36%-50% of patients with PG suffer from IBD. PG appears as a tender erythematous papule evolving into a livid pustule with central necrosis and subsequent ulceration, occurring in single or multiple lesions. The ulcer often has an irregular outline and is sharply demarcated with a heaped-up mushy violaceous border, surrounded by a erythematous zone (Figure 1). Often minor trauma, needle stitches or biopsy can induce new PG lesions



Figure 2 Erythema nodosum in a patient with CD.



Figure 3 Pyoderma gangrenosum in a patient with CD.

(pathergy phenomenon). PG lesions have a predilection for the lower limbs but may occur in any area of the skin, sometimes even as peristomal ulcers^[57]. The diagnosis of PG is made clinically; nevertheless skin biopsy of the border of the ulcer may be performed to rule out vasculitis or infection. There are no pathognomonic histologic features, generally revealing only diffuse neutrophilic infiltration and dermolysis.

PG is the most severe skin manifestation in IBD. PG is painful and often persisting despite adequate therapy. Without treatment, PG can last for years and ulcers may spread. Therefore, aggressive and early treatment is required. Local wound care consists of dressings, mild débridement of necrotic material and eschar (continuous wet saline compressions, topical enzymatic ointment, hydrocolloid dressings similar to common ulcer treatment). Topical tacrolimus has also emerged as potentially useful therapy^[58]. First line systemic treatment in PG is high dose prednisolone. Intravenous pulse therapy over three days is highly effective. Careful tapering should be started with clinical improvement. In steroid dependent or steroid refractory (no improvement within 5 d) cases, immunosuppressive therapy should be initiated. In mild cases a combination of steroids with dapsone has been successfully used with an initial dosage of dapsone 100 mg po/d, gradually increasing to 200-300 mg/d^[59]. In more severe cases cyclosporine or tacrolimus are effective. Steroid dependent patients require immunosuppressive treatment with azathioprine/6-mercaptopurine. A variety of other treatments (thalidomide, topical cromoglycate,



Figure 4 Sweet syndrome: papulosquamous exanthema in a patient with UC.

clofazimine, plasmapheresis, granulocyte apheresis, hyperbaric oxygen) have been reported anecdotally. Surgical intervention should be avoided, if possible, since it may induce pathergy. In resistant cases infliximab at a dose of 5 mg/kg has been used successfully^[59,60]. PG often takes a prolonged course, but in general will be controllable with medical therapy. About 35% of patients will experience relapsing PG.

Oral ulcerations

Oral aphthous ulcers occur in at least 10% of patients with UC and 20%-30% with CD and rapidly resolve once remission is achieved. Stomatitis, as an adverse event of methotrexate therapy, should be taken into account.

Miscellaneous skin lesions

Many other skin affections have been described in patients with IBD, such as Sweet's syndrome (neutrophilic dermatosis)^[61] (Figure 4), leukocytoclastic vasculitis, psoriasis, epidermolysis bullosa acquisita, and cutaneous polyarteriitis nodosa. Since these diseases have been mainly reported as single case reports, they probably occur coincidentally rather than as a true EIM. Angular cheilitis in nearly 8% of patients with CD often is a sign of iron deficiency.

EYE MANIFESTATIONS

Two to five percent of patients with IBD experience ocular manifestations^[62]. The manifestations range from conjunctivitis to more significant inflammation, including iritis, episcleritis, scleritis and anterior uveitis. Mild cases of conjunctivitis may be diagnosed clinically, but in other cases early referral to an ophthalmologist is important for accurate diagnosis.

Episcleritis

Episcleritis is less common in UC than in CD, presents as an infection of the ciliary vessels and an inflammation of the episcleral tissues and does not affect visual acuity. Inflammation episodes tend to occur in association with active bowel disease. Successful treatment consists of both

topical corticosteroids and treatment of the underlying bowel disease. Scleritis affects deeper layers of the eye and can cause lasting damage if untreated.

Uveitis

Uveitis is less common than episcleritis and occurs in 0.5%-3% of patients. It does not affect visual acuity unless it involves the posterior segment. Uveitis frequently presents bilaterally, is insidious in onset and chronic in duration. It is more common in females and may not parallel bowel disease activity. On slit-lamp examination uveitis presents as a perilimbal edema and "inflammatory flare" in the anterior chamber. Conjunctival vessel injection and corneal clouding may also be seen. An acute episode of uveitis can lead to permanent damage of the eye with iris atrophy, lens deposits or synechiae. More aggressive therapy may be necessary, especially when the posterior chamber is affected. Prompt diagnosis and therapy with topical and systemic steroids is crucial and sometimes intraocular injections of corticosteroids may be necessary. Steroid treatment should be continued for four weeks before tapering if uveitis is under control. Iridospasm is relieved by topical mydriatic eyedrops. Successful treatment for IBD-associated uveitis with infliximab was first described in one CD patient having a suitable extraintestinal constellation (uveitis and sacroileitis)^[63]. In rheumatoid arthritis with severe refractory uveitis, infliximab was effective but with an unexpected high rate of side effects^[64].

Ocular complications in IBD include keratopathy and night blindness resulting from malabsorption of vitamin A. Cataract development is a severe side effect of steroid therapy, therefore regular ophthalmologic examination should be considered. Rare eye manifestations are retinal vascular disease (central vein occlusion or vasculitis), peripheral corneal ulcers, corneal infiltrates and central serous chorioretinopathy with bullous retinal detachment.

HEPATOBIILIARY MANIFESTATIONS

Primary sclerosing cholangitis

In UC, the main hepatic EIM is primary sclerosing cholangitis (PSC), a chronic inflammatory disease of the biliary tree, occurring in approximately 3% of all patients (Figure 5). The diagnosis is made by endoscopic or magnetic resonance cholangiography, showing beading, irregularity, and stricturing of intrahepatic and extrahepatic ducts. Histology ranges from obliterating concentric fibrosis of the bile ducts to chronic inflammatory infiltrates in the portal tracts resulting in interface hepatitis. Low titres of autoantibodies against smooth muscle, parietal cells, and nuclear antigens are common, and high titres of autoantibodies to neutrophils (p-ANCA)^[65], showing a perinuclear pattern of staining, have been described. The majority (70%) of patients have the HLA-DR3, B8 haplotype.

Treatment

Ursodeoxycholic acid has been suggested to delay disease progression^[66]. Although not avoiding the progression of liver disease, ursodeoxycholic acid has been demonstrated



Figure 5 ERC demonstrating primary sclerosing cholangitis in a patient with CD.

to exhibit chemopreventive effects and reduce the risk of colorectal dysplasia in ulcerative colitis^[67,68]. This is of importance since patients with UC and concomitant PSC have a significantly higher risk of developing colorectal neoplasia compared with patients having UC only^[69]. PSC is seen as a premalignant condition for the development of cholangiocarcinoma as well.

Early symptomatic treatment of cholestasis is mandatory to avoid septic complication and consists of endoscopic dilatation with or without stent insertion. PSC is slowly progressive and independent of the course of UC, developing the complications of portal hypertension and chronic liver failure. For end-stage liver disease, liver transplantation remains an option.

PSC may occur in 4% of patients with CD as well, usually in those with colonic disease. Inflammatory changes of the small ducts show a normal cholangiogram and pericholangitis on liver biopsy^[70].

Hepatobiliary complications

Gallstones are frequent in patients with CD, of which 25% develop gallstones mainly due to malabsorption of bile salts from the inflamed terminal ileum. Abnormalities of liver function (elevated serum aminotransferase and alkaline phosphatase) are common in patients with malnutrition, sepsis, and fatty liver due to severe attacks of IBD. These correlate with disease activity and return to normal once remission is achieved. Factors favouring fatty infiltration of the liver in severely ill patients are poor nutrition and often concomitant steroid therapy.

PANCREATIC MANIFESTATIONS

There is an increased risk for acute as well as chronic pancreatitis in IBD^[71-73] documented by a multitude of case reports and case series.

Acute pancreatitis

Especially in cases of acute pancreatitis, it may be difficult to determine the true incidence of EIM^[74]. Many drugs in IBD treatment have the potential to induce acute pancreatitis (e.g. salicylates, azathioprine and 6-mercaptopurine, rarely corticosteroids). Drug-induced pancreatitis typically occurs within the first weeks after

commencing drug-therapy^[75], the course is usually mild and resolves quickly after discontinuing the drug. Especially in CD regional inflammatory complications due to duodenal/papillary involvement or biliary complications should be considered. Nevertheless, after excluding extraintestinal complications, an increased risk for idiopathic pancreatitis remains in patients with IBD (1%-1.5%) that should be considered as true extraintestinal involvement.

Pancreatic autoantibodies have been found in up to 40% of CD patients^[76]. In contrast to earlier reports^[77] the prevalence seems to be increased in both CD and UC patients as well as in first-degree relatives^[78]. The relevance for disease is still unclear, however. In a series with 64 antibody-positive CD patients the proportion suffering from exocrine insufficiency was 27% compared to 8% in antibody negative patients^[79]. Further studies are needed to show whether these antibodies, apart from their diagnostic relevance for IBD, actually play a role in pathogenesis and whether they can help to identify patients at risk of developing an EIM involving the pancreas.

Chronic pancreatitis

Endoscopic retrograde pancreatography (ERP) is still the most sensitive and specific test for chronic pancreatitis. There are several reports on intrapancreatic duct changes in patients with IBD^[80,81]. Pancreatic function has been investigated as well. In the study of Heikius, the exocrine function was found to be decreased in only 4% of patients using the secretin test. These results are consistent with two other studies that diagnosed exocrine insufficiency in a minority of IBD patients only^[73,82]. In all studies, chronic alcohol ingestion as a potential cause for chronic pancreatitis had been excluded. The pathogenesis of chronic pancreatitis in patients with IBD remains unclear. Since there has only been one documented case of pancreatic granuloma in Crohn's disease^[83], pancreatitis has been considered to be caused by circulating inflammatory mediators rather than directly-involved pancreatic tissue. As discussed above, autoantibodies against pancreatic tissue may play a role in the development of exocrine insufficiency.

THROMBOEMBOLIC EVENTS

Patients with IBD are at increased risk of developing thromboembolic complications. The incidence of deep vein thrombosis and pulmonary embolism is increased and is a major cause of mortality in IBD. Venous thrombembolism is more common than arterial embolism. Conventional risk factors, such as prolonged immobilization, hospitalization, sepsis, surgery or invasive procedures, contribute to this increased risk especially in active or complicated IBD. In severe disease, thrombocytosis and increased concentrations of many clotting factors that behave as acute phase proteins, lead to a procoagulatory status. The majority of IBD patients with thromboembolic events have active disease^[84]. However, a recent study demonstrated nicely that IBD, as such, is a risk factor for thromboembolic events. Comparing patients with IBD, rheumatoid arthritis and celiac disease to age and sex-matched controls, they found that patients

with IBD have a 3.6-fold increased risk of experiencing thromboembolic events in contrast to the other chronic inflammatory diseases that have no increased risk^[85].

There is no consistent evidence that inherited thrombophilia is associated with IBD. The main established genetic risk factors have not been found to be increased in IBD^[86-88]. In more than half of patients with thrombosis, no predisposing factor is evident^[86]. Therefore, screening for these risk factors seems to be justified only in the case of a personal or a family history of thromboembolism. Hyperhomocysteinemia, more common in IBD patients than in controls, is associated with an increased risk of thromboembolism as well. To date it is controversial if polymorphisms in the methylenetetrahydrofolate reductase (MTHFR) gene leading to hyperhomocysteinemia are more frequent in IBD patients compared to healthy controls^[87,89,90]. Lack of Vitamin B6, B12 or folate or the use of folate-inhibiting drugs (methotrexate, sulfasalazine) can contribute to acquired hyperhomocysteinemia.

Corticosteroid treatment may cause a hypercoagulable state with increased risk of thrombosis, but systemic data pointing towards a significant association is missing. In general, all hospitalized patients with IBD should receive low-dose heparin for thromboprophylaxis unless there is severe bleeding.

MANIFESTATIONS OF THE KIDNEYS AND THE URINARY TRACT

Glomerulonephritis

There are several reports on glomerulonephritis in patients with IBD. Many kinds of histology and clinical course, ranging from minimal change nephropathy to rapidly progressive glomerulonephritis, have been described^[91-94]. A true extraintestinal manifestation is difficult to prove, however, and the combination of glomerulonephritis and IBD may well be coincidental.

Interstitial nephritis and tubular proteinuria

Again, it is difficult to make a distinction between EIM and side effects of medication. Single cases of interstitial nephritis have been reported in IBD. At least one case of granulomatous interstitial nephritis has been attributed to underlying CD^[95], thus interstitial nephritis may represent a EIM of IBD. Medication, especially therapy with aminosalicylates, may cause interstitial nephritis as well. However, surveillance data from France^[96] and the UK^[97] report a low incidence of renal impairment under salicylate therapy. An additional, large epidemiologic study recently demonstrated that renal impairment is attributed to severity of the underlying IBD rather than to 5-ASA treatment^[98]. This is in accordance with the fact that the frequently observed tubular proteinuria in IBD is related to disease activity and not dependent on 5-ASA treatment^[99-101]. The clinical relevance of this proteinuria remains to be determined.

Renal and urinary complications

In patients with CD, uric acid and oxalate stones are common. Due to fat malabsorption luminal calcium is

bound to free fatty acids. Lack of free calcium results in increased oxalate absorption, hyperoxaluria and formation of renal oxalate stones. Hyperoxaluria may cause chronic tubulointerstitial nephritis as well. Prevention treatment of oxalate stones consists of low oxalate diet and supplementation of oral calcium (1-2 g/d). The risk for uric acid stones is increased with volume depletion (e.g. due to diarrhoea, ileostomy) and a hypermetabolic state in critically ill patients. Prevention of uric acid stone recurrence consists of hydration and oral urinary alkalization.

A rare complication is renal amyloidosis (AA-Amyloidosis) due to chronic inflammation; it mainly occurs in CD and is rarer in UC. Systemic AA amyloidosis is caused by extracellular deposition of fragments of the circulating acute-phase-plasma protein (SAA). Usually the mean duration between onset of the underlying chronic disease and the occurrence of amyloidosis is at least 15 years. The diagnosis is confirmed by detection of amyloid (Congo red staining) deposits in tissue (rectum biopsy, abdominal fat aspiration). Progression of amyloidosis can be stopped by controlling inflammation. Additional genitourinary complications may be caused by (enterovesical) fistula formation, perivesical abscesses, cystitis and obstructive uropathy.

PULMONARY MANIFESTATIONS

Various pulmonary manifestations have been reported in IBD including small and large airway dysfunction as well as obstructive and interstitial lung disorders. Case reports do not show a uniform picture of disease. Various entities including bronchial hyperreagibility, bronchitis and bronchiectasis, inflammatory tracheal stenosis, interstitial pneumonitis as well as bronchiolitis obliterans organizing pneumonia (BOOP) have been described. The respiratory symptoms usually follow the onset of the IBD. Sometimes pulmonary disease, especially serositis, correlates with IBD activity. Parenchymal lung disease often develops independently of disease activity. Interestingly, colectomy seems to be a risk factor for developing pulmonary symptoms with a frequent postoperative onset^[102,103].

Recently, a large population-based Canadian study reported airway disease as the most common concomitant chronic disease in patients with CD and second most common in patients with UC^[3]. Patients with CD and with UC were more likely, 30%-40% and 50%-70% respectively, than controls to have asthma^[3]. The high frequency of pulmonary involvement in IBD probably reflects the commonality of bronchus-associated and gut-associated lymphoid tissue^[104]. Subclinical lung involvement is much more common than apparent respiratory symptoms, reported in as many as 30%-60% of patients with CD. Bronchoalveolar lavage reveals alveolitis in as much as 50% of CD patients without any clinical respiratory symptoms^[105] and abnormal pulmonary function tests have been reported in 42% of IBD patients without respiratory symptoms compared to only 3% in controls^[106]. Interestingly, these changes persist during remission.

Persistent airway inflammation can result in airway narrowing, dependent on the localization, resulting in

tracheal stenosis, bronchiectasis or bronchiolitis obliterans. In fact, the majority of patients with IBD-related respiratory manifestations present with chronic bronchitis or bronchiectasis^[102]. Chest X-rays are often unpecific; high resolution chest CT scanning may demonstrate bronchial wall thickening, dilated airways, branched opacities due to mucoid impaction^[107]. In lung biopsy nonspecific inflammation, small airway fibrosis and sometimes granulomatous bronchiolitis have been reported^[108,109]. The most common interstitial lung disease associated with IBD is bronchiolitis obliterans organizing pneumonia (BOOP)^[102]. Chest X-ray reveals patchy focal opacities or diffuse infiltrates, while CT scanning often demonstrates pleural-based opacities and air bronchograms. Typical granulomatous interstitial lung disease in Crohn's disease is rare and may mimic sarcoidosis with the occurrence of noncaseating granulomas and elevated CD4/CD8 ratios in bronchoalveolar lavage fluid^[110-112]. Apart from airway inflammation and interstitial lung disease other pulmonary manifestations of IBD include serositis^[113]. Serositis occurs as exudative pleural effusion pericarditis, pleuropericarditis and myocarditis^[107].

Complications

Pulmonary parenchymal involvement may be related to IBD, but also may be induced by drugs (e.g. mesalazine, sulfasalazine, methotrexate). Many cases of drug-induced pulmonary complications in IBD patients have been reported. Salicylates of all kinds can induce different types of interstitial lung disease, such as BOOP and granulomatous lung disease. The pulmonary toxicity of 5-aminosalicylic acid (5-ASA) is less common than with sulfasalazine, but pulmonary infiltrates, sometimes eosinophilia or BOOP may develop as well^[102,114,115]. Pulmonary infiltrates with eosinophilia (PIE syndrome) can occur with or without the use of sulfasalazine or mesalamine^[116]. Chest X-rays often show peripheral infiltrates typical of chronic eosinophilic pneumonia and laboratory values reveal eosinophilia. Methotrexate treatment may cause serious hypersensitivity pneumonitis or pulmonary fibrosis. Chest X-rays show diffuse alveolar or interstitial infiltrates.

Especially if patients receive combination immunosuppression, the possibility of opportunistic pulmonary infections should be taken into account.

Treatment

It is unclear whether asymptomatic patients should receive therapy at all. However, it should be born in mind that IBD patients carry an increased mortality risk due to pulmonary disease^[117-119]. Depending on the type of pulmonary complication, inhaled or systemic steroid therapy may be effective. In various forms of airway inflammation, inhalatory steroid therapy (e.g. beclomethasone up to 1200 µg/d) is generally effective, with large airway inflammation being more responsive than bronchiolitis. Patients with bronchiectasis are less likely to respond to inhaled steroids and may require oral steroids. In severe airway inflammation with upper airway obstruction, such as subglottic involvement, intravenous

steroids may be required. Interstitial lung disease usually requires systemic steroids; e.g. prednisolone 0.5-1.0 mg/kg per day for a longer period of time, usually months, depending on the clinical course.

CARDIAC MANIFESTATIONS

Pericarditis or perimyokarditis with or without an effusion have been described in a few case reports on patients with active IBD. Again, it may be difficult to rule out drug toxicity since salicylates are potential culprits. However, in the Canadian population-based study, both patients with CD and UC were at an increased risk of developing pericarditis in comparison to healthy controls^[3]. Age-adjusted prevalence ratios were 3.07 in CD and 3.33 in UC, respectively, although the numbers were small^[120].

NEUROLOGICAL MANIFESTATIONS

The association of autoimmune neurological disorders and IBD has long been hypothesized, especially for multiple sclerosis^[121-125]. Furthermore there are some sporadic reports of neuropathies in IBD such as optic neuritis^[126], peripheral neuropathies^[127] and (subclinical) sensorineural hearing loss^[128,129]. MRI studies have shown clinical nonapparent cerebral white matter lesions in patients with IBD^[130]. A recent retrospective cross-sectional study evaluated the risk of patients with IBD to develop either multiple sclerosis, optic neuritis or demyelinating disease in the pre-anti-TNF- α era^[131]. The authors found a small but significant association of IBD with these demyelinating diseases (OR 1.67). The risk was not found to be related to the use of steroids and/or immunosuppressive therapy with thiopurines. These results are of special interest in the light of recent reports on new unexpected adverse events of biological agents.

Recently, alarming reports on the development of progressive leucoencephalopathy under treatment with natalizumab, an antibody directed against α 4-integrin, have resulted in stopping all ongoing trials with this substance^[132,133]. Similar observations exist on the anti-TNF α -therapeutic agents infliximab, etanercept and adalimumab^[134-136] and have led to additional safety warnings concerning the use of these drugs. To date, patient numbers are too small to draw final conclusions but these agents may well accelerate the preexisting risk for demyelinating disorders in IBD patients.

Metronidazole, often used in fistulizing CD, can cause polyneuropathy if given as long term treatment. Nutritional deficiencies (Vitamin B12) should always be taken into consideration and looked for especially in long-lasting and severe disease.

SUMMARY

In conclusion, IBD are systemic disorders and not restricted to the intestine. Recent reports on the involvement of new organ systems emphasize that almost every site of the body can be affected by the inflammatory process. The possibility of contributing

Table 1 Extraintestinal symptoms in inflammatory bowel disease

Extraintestinal manifestations	Extraintestinal complications
Musculoskeletal diseases	
Peripheral Arthritis	Drug-induced osteoporosis and osteonecrosis
Ankylosing spondylitis	Bacterial infection of joints (fistulization, immunosuppression)
Isolated sacroiliitis	Septic arthritis
Metastatic Crohn's disease	
Mucocutaneous diseases	
Erythema nodosum	Anal fissures
Pyoderma gangraenosum	Fistulas
Aphthous stomatitis/oral ulceration	
Psoriasis?	Acrodermatitis enteropathica (Zinc deficiency)
Epidermolysis bullosa acquisita?	Purpura (Vitamin C and K deficiency)
Sweet Syndrome?	Glossitis (Vitamin B or Zinc deficiency)
Erythema exsudativum multiforme?	Hair loss (protein deficiency)
	Brittle nail (protein deficiency)
	Perleche (Iron deficiency)
	Candidiasis (Zinc deficiency, immunosuppression)
	Mucositis/stomatitis (methotrexate)
	Drug-induced rash, allergic exanthema
	Moon-face, acne, stretch marks, skin atrophy (steroid treatment)
Ocular diseases	
Anterior Uveitis	Night blindness Vitamin A deficiency
Conjunctivitis	Kerathopathy Vitamin A deficiency
Iritis	Opportunistic infections (immunosuppressants)
Skleritis/Episcleritis	
Hepatobiliary diseases	
Primary sclerosing cholangitis	Gall stones in Crohn' disease
Granulomatous Crohn's hepatitis	Fatty liver
Autoimmune chronic active hepatitis?	
Biliary cirrhosis?	
Pancreatic diseases	
Acute pancreatitis	Drug-induced pancreatitis (5-ASA, azathioprine)
Chronic pancreatitis, exocrine insufficiency	Biliary pancreatitis in Crohn's disease
	Duodenal involvement in Crohn's disease
Blood and vascular diseases	
Thrombembolic events	Anemia (Iron-, folate-, vitamin B12-deficiency)
Autoimmune hemolytic anemia?	Thrombocytosis, leucocytosis
Thrombocytopenic purpura (Moschowitz Syndrome)?	Hypercoagulation: venous thrombosis, thrombembolism
Renal diseases	
Tubular proteinuria	Nephrolithiasis (oxalate stones, uric acid stones)
Glomerulonephritis?	Local affection involving the urethro-genital system
	Acute interstitial nephritis (drug related SASP, 5-ASA)
Interstitial nephritis?	Drug-induced renal insufficiency (5-ASA, SASP, cyclosporine)
	Renal amyloidosis
Bronchopulmonary diseases	
Chronic bronchitis/bronchiolitis/bronchiectasis	Drug-induced hypersensitivity pneumonitis
Acute laryngotracheitis/tracheal stenosis	Drug related pulmonary fibrosis (methotrexate)
Bronchiolitis obliterans organizing pneumonia	Drug-induced pleuritis
Pleuritis/serositis	Opportunistic infections (immunosuppression)
Cardiac diseases	
Pericarditis	Drug-induced pericarditis (5-ASA)
Myocarditis?/perimyocarditis	
Neurological diseases	
Demyelinating diseases including multiple sclerosis	Peripheral neuropathy (Vitamin B12 deficiency)
Optic neuritis, sensorial hearing loss	Drug-induced leucoencephalopathy (natalizumab, infliximab)
Myasthenia gravis?	Drug-induced polyneuropathia (metronidazole)

disease complications, especially through the side effects of treatment, should always be born in mind. The distinction between disease and treatment side effects can be extremely difficult and may sometimes be impossible. Recent reports on multiple organ involvement, including the central nervous system, point out the importance of an increased awareness for these potential problems. In addition, these data provide additional warning concerning the use of the new biological treatment strategies. These agents have to be prescribed with care since long term experience with toxicity is limited.

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GASTRIC CANCER

Clinical profile of gastric cancer in Khuzestan, southwest of Iran

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relatively long delay. Most patients present in advanced stages, which favors a poor overall survival. Family history of GC has a significant problem in our area. Studying the etiology of this cancer in south Iran and earlier diagnosis and subsequent better cares are recommended.

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Key words: Gastric cancer; Epidemiological features; Khuzestan; Southwest of Iran

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Abstract

AIM: To analyze the characteristics of epidemiological, clinical and survival patterns among patients with carcinoma of the stomach.

METHODS: We retrospectively studied the characteristics of 186 gastric adenocarcinoma patients at Ahwaz Jundishapur University Hospitals (AJSUH) from September 1, 1996 to September 1, 2002. All the patients had histopathologically-confirmed malignancy. Demographic variables, family history of gastric cancer (GC), clinicopathologic characteristics and treatment-related variables were analyzed. Univariate analysis was performed with the log-rank test and multivariate analysis with Cox regression. $P < 0.05$ was considered statistically significant.

RESULTS: Male to female ratio was 2.6:1. The mean age was 60.6 years and 14% of the patients were younger than 40 years. Adenocarcinoma, gastric lymphoma, and gastric metastasis were found in 94.5%, 2.3%, and 3% patients, respectively. There was an average of 6-mo delay between the initial symptoms and the diagnosis. Among adenocarcinoma groups, intestinal type was the commonest (55.9%) and the distal third was the most common localization (88.4%). One hundred and thirty-four patients (72.1%) were males. Thirty-one patients (17%) had a family history of GC. Surgery was performed in 90% of patients (non-curative).

CONCLUSION: The epidemiological features of GC in south Iran mimic those in high-risk areas. There is a higher frequency of GC in young patients at our institution. Patients are detected and treated after a

INTRODUCTION

Gastric cancer (GC) remains the third most common malignancy in the world^[1]. The pattern and incidence of GC vary widely in different parts of the world. Costa Rica and Japan have the first and second highest rates in the world with a rate of 77.5 and 50.5/100000 persons, respectively^[2,3]. Gastric cancer is the most common malignancy in Iran and its incidence is particularly high in the Ardabil Province in the northwest of the country. In this province, the age standard incidence rate is 49.1 and 25.4/100000 persons per year in males and females, respectively. The cause of the high incidence of GC in this geographical region is unknown^[4,5].

The epidemiology of GC has been widely studied in the Western world as well as in Japan^[6-9]. However, only few scattered reports from the developing world have been published^[10-14]. There is a lack of good descriptive data on GC from the Middle East countries, where both cancer registration and prevalence of risk factors are relatively unknown. Because of the decreasing trend taking place in the Western world as a result of possibly socio-economic development and its consequences, it is important to gain an insight into what is happening in other parts of the world such as in the Middle East. This prompted us to report the epidemiological and clinicopathological features of gastric malignancy in Khuzestan, southwest of Iran in comparison to other countries whenever possible. This

could assist in the better understanding of the important risk factors, which contribute to the development of GC. This also gives a clue about whether or not screening programs are needed in our region.

MATERIALS AND METHODS

We retrospectively analyzed the clinicopathologic characteristics of 186 gastric adenocarcinoma patients who were admitted to or operated upon at Ahwaz Jundishapur University Hospitals (AJSUH) from September 1, 1996 to September 1, 2002. Age, sex, method of operation, size of lesion, location of cancer in the stomach and stage were analyzed in patients, retrospectively. Histologically confirmed primary gastric malignancy was found in 186 patients, including 177 patients with adenocarcinoma, 5 patients with primary gastric lymphoma (PGL), and 3 patients with malignant gastric metastasis. All available endoscopy reports were reviewed. At the entry, clinical symptoms, demographic data and medical history were recorded and gastroscopy was performed to establish the endoscopic diagnosis and status of *H pylori* infection. During the examination of biopsy specimens from the stomach, silver or modified Giemsa staining and histological examinations were performed to establish the diagnosis and status of *H pylori* infection.

Patients and/or family members were contacted. Gastric adenocarcinoma was classified into intestinal type (IT), diffuse type (DT) or mixed type according to the histological criteria of Lauren^[15]. Tumor staging in each patient was based on clinical information, preoperative radiological investigations, operative findings and pathological examination. The staging was made in accordance with the TNM system^[16].

Clinicopathologic data were compared using the χ^2 and Fisher's exact tests. $P < 0.05$ was considered statistically significant.

RESULTS

During the study period, 186 patients with GC were identified, 127 (68.3%) patients were males with a male to female ratio of 2.6:1. The peak incidence was in the age group of 60-69 years (40%), followed by the age group of 50-59 years (16%). Approximately 14% of the patients were younger than 40 years and 6% of the patients were younger than 30 years. The mean age for the whole group was 63 years (range 21-91 years). Table 1 shows the age distribution in the patient groups.

Features of the patients are summarized in Table 2. There was an average of 6-mo delay (range 1-13 mo) between the initial symptoms and diagnosis.

Carcinomas were located most frequently in the lower third of the stomach, accounting for 88.5% (165/186) of all patients. Table 3 shows the distribution of various histological types of gastric adenocarcinoma according to the sites that were affected. One hundred (53.5%) and eighty-six (46.5%) patients lived in urban and rural areas, respectively. The histopathology of gastric biopsy showed *H pylori*-associated chronic active gastritis in 166 (88.9%) patients.

Table 1 Age distribution in patient groups

Age (yr) group	Male patients		Female patients	
	(n)	%	(n)	%
1-10	0	0	0	0
10-19	0	0	0	0
20-29	4	2.15	7	3.76
30-39	15	8	7	3.76
40-49	11	6	6	3.2
50-59	20	11	10	5.4
60-69	50	27	24	12.9
70-79	19	10	6	3.2
≥ 80	7	3.7	0	0
Total	126	67.9	60	32

Table 2 Features of the studied patients

Signs and symptoms	Patients (n)	%
Abdominal pain	93	50
Weight loss	23	12
Dyspepsia	91	48.8
Nausea, vomiting	74	40
Abdominal mass	4	2
Anorexia	182	97.7
Dysphagia	30	16
Gastrointestinal bleeding	26	14
Ascites	28	15
Constipation	5	2.5

Table 3 Distribution of gastric adenocarcinomas according to their site n (%)

Histopathological type	GEJ and gastric cardia	Gastric corpus and antrum	Total
Intestinal type adenocarcinoma	24 (12.9)	80 (43.1)	104
Diffuse type adenocarcinoma	14 (7.5)	60 (32.3)	74
Gastric lymphoma and metastasis	0 (0)	8 (4.3)	8
Total	38 (16.7)	148 (79.9)	186

GOJ: Gastro-esophageal junction.

According to TNM staging, the proportions of stages I A, I, II + III, and IV in the studied groups were 0% (0/186), 28% (53/186), and 71% (133/186), respectively (Table 4).

DISCUSSION

Gastric cancer is the most prevalent malignancy in Iran. If GC is diagnosed at an early stage, patients can have a highly favorable prognosis and avoid extended surgery, which may produce complications, especially in the elderly people. However, the symptoms of GC are non-specific and vague, when symptomatic patients experience epigastric pain and discomfort and definitive symptoms such as weight loss or obstructive symptoms and metastases that often impede curative radical resection. Additionally, the results of GC treatment do not differ

Table 4 TNM stage and methods of operation in patient groups

Stage (TNM) and methods of operation	Patients (n)	%
I	0	10
II	20	10.8
III	33	17.5
IV	133	71
Curability		
Curative resection	15	8
Palliative resection	95	51
No resection	76	41
Type of resection		
Total and subtotal gastrectomy	70	37.6
Distal gastrectomy	14	7.5
Other resections and palliative operation	26	14

markedly from the past results though there are improved surgical techniques and adjuvant treatments. Researchers have shown that the prognosis of GC has not changed in the past 20 years^[9,17]. The only method that is likely to improve the survival rates is early detection of GC. Our patients tended to present late as evidenced by the fact that there was a long interval between the onset of symptoms and presentation. There was an average of 6-mo delay (range 1-13 mo) between the initial symptoms and the diagnosis.

This is not due to the insufficient current endoscopic services, but due to the fact that many people in our area who have dyspeptic symptoms visit non-specialist physicians who either prescribe medications for long term treatment or use drugs in order to ameliorate the pain. Subsequently, some of these patients whose cause of dyspepsia is cancer present with late stage GC or one of its complications. In addition, the elderly people usually fail to make use of the available medical services. However, general practitioners should be more liberal in referring patients for endoscopy and resist the temptation to treat dyspeptic patients with anti-ulcer therapy without endoscopy, especially in elderly people and in patients with alarming signs. Open access endoscopy, greater efforts in patients' education and improvement of the diagnostic technical skills may improve the early detection of GC.

According to TNM staging, the proportions of stages I A, I, II + III, and IV in the studied groups were 0% (0/186), 28% (53/186), and 71% (133/186), respectively. Approximately more than two-thirds of the patients were diagnosed with advanced GC.

These results re-emphasize that GC symptoms are non-specific and need an early screening examination. A public screening system for gastric cancer has not yet been introduced in Iran and in our area.

Gastric cancer is the most common malignancy in Iran and its incidence is particularly high in the Ardabil Province in the northwest of the country^[12,13,18]. In this province, the age standard incidence rate is 49.1 and 25.4/100 000 persons per year in males and females, respectively.

The cause of the high incidence of gastric cancer in our country is unknown.

The following two factors may play a role. First, the rapid change in dietary habits constitutes a risk factor.

Vitamin C-rich fresh vegetables and fruits, starch and natural unprocessed wheat products are the major constituents of Iranian food. However, canned food, hot spices, pickles and animal proteins are now dominating the Iranian menu.

Fermentation of foods involves the production of nitrosamine. This compound has been implicated as a risk factor for GC in many studies and frequent consumption of fermented food may be a risk factor for GC in our region because our people use such compounds most often.

However, to further investigate the association between fermentation and GC, more comprehensive and detailed data are required. Salt has been indicated as a risk factor for GC in many previous studies. Since the use of salt and fermentation in our regional food preparations is strongly inter-related, we are unable to clearly separate the independent effects of the two variables.

It is known that the environmental risk factors for GC are dietary in origin^[19,20].

In our region the incidence of *H pylori* infection (> 80%) is high and there is a substantial incidence of reflux disease. In addition, 30% people smoke, < 5% people drink alcohol and 60% people have a body mass index > 25^[21].

Most resections are done with palliative intent. The low rate of gastrectomy with "curative" intent could be explained by the high proportion of patients with advanced GC at presentation.

Patterns of GC in our area are similar to those reported from high-risk regions worldwide^[22]. In our study, the male to female ratio was 2.6:1, the peak incidence was in the age group of 60-69 years (40%), followed by the age group of 50-59 years (16%). Approximately 14% of the patients were younger than 40 years and 6% of the patients were younger than 30 years. The mean age of the whole group was 63 years (range 21-91 years). Among our study groups intestinal type (IT) adenocarcinoma was the commonest histological subtype (56%).

IT adenocarcinoma was more common than DT adenoma (1.65: 1) and distal location was more frequent than the proximal one (4.77:1) (Table 1).

In Western countries, PGL and metastasis are represented only in 2%-5% of gastric malignancies^[23]. It was 4.3% in our series, which was lower than 9% from neighboring Iraq^[24], and 14%-22% from Saudi Arabia^[11,25]. During the past three decades the site of PGL in the Middle East has changed. Small intestinal involvement has become less common and gastric involvement more frequent. This varying pattern seems to be environmental in origin. The ratio of PGL to gastric adenocarcinoma among our patients was 0.045, which is similar to the western series^[26].

In conclusion, several symptoms of GC are non-specific. The majority of identified gastric adenocarcinoma patients are symptomatic, and have a lesser chance of being cured by operation and a lower survival rate. The patients with dyspeptic symptoms and alarming signs should be referred to earlier diagnostic endoscopy. Improvements in diet and food storage and control of *H pylori* infection, by indirect means such as improving the

general sanitary conditions or by direct interventions such as eradication are likely to offer great potentials for the prevention of GC in this area.

Although this study has highlighted the pertinent epide-miological and clinicopathological features of gastric malignancy in Khuzestan Province in Iran, further studies are needed to evaluate the environmental risk factors, incidence, the treatment outcomes and survival rate.

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VIRAL HEPATITIS

HepG2 cells support viral replication and gene expression of hepatitis C virus genotype 4 *in vitro*

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cluster was undetectable in uninfected HepG2 cells.

CONCLUSION: HepG2 cell line is not only susceptible to HCV infection but also supports its replication *in vitro*. Expression of HCV structural proteins can be detected in infected HepG2 cells. These cells are also capable of shedding viral particles into culture media which in turn become infectious to uninfected cells.

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Key words: Hepatitis C virus; *In vitro* propagation; Genomic replication; Gene expression; HepG2 cells

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Abstract

AIM: To establish a cell culture system with long-term replication of hepatitis C virus (HCV) genome and expression of viral antigens *in vitro*.

METHODS: HepG2 cell line was tested for its susceptibility to HCV by incubation with a serum from a patient with chronic hepatitis C. Cells and supernatant were harvested at various time points during the culture. Culture supernatant was tested for its ability to infect naïve cells. The presence of minus (antisense) RNA strand, and the detection of core and E1 antigens in cells were examined by RT-PCR and immunological techniques (flow cytometry and Western blot) respectively.

RESULTS: The intracellular HCV RNA was first detected on d 3 after infection and then could be consistently detected in both cells and supernatant over a period of at least three months. The fresh cells could be infected with supernatant from cultured infected cells. Flow cytometric analysis showed surface and intracellular HCV antigen expression using in house made polyclonal antibodies (anti-core, and anti-E1). Western blot analysis showed the expression of a cluster of immunogenic peptides at molecular weights extended between 31 and 45 kDa in an one month old culture of infected cells whereas this

INTRODUCTION

The lack of an efficient cell culture system or a readily available small animal model has hampered the development of therapies for hepatitis C virus (HCV) infection. The chimpanzee is the only animal that is susceptible to hepatitis viral infections, but its endangered status and financial considerations limit its widespread use in viral hepatitis research. Despite these difficulties, recent introduction of heterologous cDNA expression systems^[1] and subgenomic replicons^[2] have allowed researchers to study various aspects of the viral life cycle and examine novel antiviral therapies. Also, among the surrogate animal models that have been developed are mouse liver repopulated with human hepatocytes and transgenic mice expressing hepatitis antigens^[3-5]. For reasons that are not evident, infection of primary hepatocytes and established cell lines with hepatitis viruses have not only produced poor viral replication and low viral yields but have also suffered from poor reproducibility^[6]. The entry of virus into a cell, followed by productive viral replication, depends on both viral and host cell proteins. Only differentiated cells may express the latter. Thus, studies of HCV and HBV infectivity initially used

primary hepatocytes from humans or chimpanzees. One group infects human fetal hepatocytes with HCV-infected serum^[7]. The viral replication is quite low and detectable only by RT-PCR amplification. Using this technique, another group showed an increase in the number of HCV+ strands by d 5, indicating that these hepatocytes support viral replication. Similarly, yet another group showed that adult primary human hepatocytes could be infected with HCV in culture conditions that support long-term cultures of hepatocytes for at least 4 mo^[8]. Under these culture conditions, viral positive-strand RNA was first detectable by PCR after 10 d of infection, and the viral RNA titer increased in culture media during a 3-mo culture. This group also demonstrated *de novo* synthesis of negative-strand viral RNA. Culture supernatants from HCV-infected hepatocytes could transmit infection to naive hepatocytes, indicating the production of infectious viral particles. However, the efficiency of viral infection is unpredictable and does not correlate with viral RNA titers. Addition of polyethylene glycol to the primary hepatocyte cultures maintained in the presence of 20 g/L dimethylsulfoxide markedly increases the infection of HBV^[9] but not HCV^[10]. HCV is lymphotropic, and peripheral blood mononuclear cell cultures support HCV replication^[11]. However, the level of viral replication is very low^[12]. Because primary hepatocytes are difficult to grow in cultures, some researchers have attempted to infect immortalized hepatocytes and hepatoma cell lines. Ikeda and colleagues^[13,14] used PH5CH, a nontumorigenic, immortalized human hepatocyte cell line, to assess the infectivity of HCV positive sera. There was an increase in the HCV sense -strand RNA during the first 12 d of culture, and the viral RNA remained detectable for at least 30 d after infection. Nucleotide sequence determination of the HCV genome in the hypervariable region 1 showed that there is a shift toward the limited HVR-1 population, indicating strong selection for HCV variants during the infection^[13]. Furthermore, IFN γ inhibits the viral replication in these cells^[14]. Recently, Guha *et al*^[5] reported that *in vitro* cell culture models can at best demonstrate the infectivity of the virus but are not suitable to study viral life cycle because of the very low levels of viral replication. These systems could be used in evaluating drugs for antiviral activity or inhibition of HCV infection. Also, Horscroft *et al*^[15] have summarized the recent development of HCV replicon cell culture system and its use in anti-HCV drug discovery. In the present study, we tested the susceptibility of HepG2 cell line to HCV and established an infection cell model that could support HCV long-term replication *in vitro*. The presence of both sense- and antisense-RNA strands and expression of viral core and envelope proteins in infected cells as well as the ability of these cells to exocytose infectious viral particles into culture media suggests that the current cellular model allows study of HCV life cycle.

MATERIALS AND METHODS

HEPG2 cell culture

Caucasian male *Homo sapiens* (human) hepatocellular

carcinoma cell line (HepG2; ATCC, HB-8065, Manassas, USA) was used to establish the *in vitro* HCV replication. HepG2 culturing and infection were carried out according to the protocols described by Seipp *et al*^[10]. HepG2 cells were maintained in 75 cm² culture flasks (greiner bio-one GmbH, Germany) containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 4.5 g/L glucose and 10 g/L L-glutamine (Bio Whittaker, a Combrex Company, Belgium) containing 100 mL/L fetal calf serum (FCS; Biochrome KG Berlin Germany), 10 g/L antibiotics (penicillin/streptomycin; Biochrome KG, Berlin, Germany) and 1 g/L antimycotic (fungisone 250 mg/L; Gibco-BRL life Technologies, Grand Island, New Y). After adding all supplements the medium is called complete. The culture medium was renewed by a fresh medium every 3 d, and cells were subcultured (6-10 d).

In summary the medium was discarded, the adherent cell layer was shortly treated with trypsin-EDTA (2.5 g/L; Sigma, Deisenhofen, Germany) to remove the left traces of trypsin inhibitors from the FCS contained in the medium. After discarding, 1.0 mL of fresh trypsin-EDTA was added onto the cells and flasks were kept either at room temperature or at 37°C (5-15 min) to observe the detachment of cells from the flask wall. To avoid extended proteolytic effect of trypsin on the detached cells complete medium was added to inhibit the enzyme activity. Cells were spun down at 400 g for 2 min, resuspended in 1 mL of complete medium, the exact count of cells was recorded in 50 μ L aliquot after mixed with equal volume of trypan blue (5 g/L; BiochromKG, Berlin, Germany.) using a hemocytometer (Right Line; Sigma, Deisenhofen, Germany). A total of 3×10^6 cells were suspended in 10 mL complete medium and incubated at 37°C in 5% CO₂.

Viral inoculation and sample collection

Cells were grown for 48 h to semi-confluence in complete medium, washed twice with FCS -free medium, then inoculated with a serum sample (500 μ L sense and 500 μ L FCS-free DMEM/ 3×10^6 cells) obtained from HCV infected patients (RT-PCR and antibody positives). The HCV genotype in the used sera was previously characterized as genotype 4 based on the method described by Ohno *et al*^[16]. The viral load in the used serum was quantitated by real time PCR and the average copy number was 290×10^6 copies/L. After 90 min, DMEM containing FCS was added to make the overall serum contents 100 mL/L in a final volume of 8 mL including the volume of human serum used for infection as mentioned above (0.04483 copies/cell). Cells were maintained overnight at 37°C in 5% CO₂. On the next day, adherent cells were washed three times with culture medium to get rid of the remaining infection serum and incubation was continued in complete medium containing 100 mL/L FCS with regular medium changes. The viral infection in HepG2 cells throughout the culture duration was assessed qualitatively by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting of viral antigens, RT-PCR amplification of sense and antisense strands and quantitatively by real time PCR.

Flow cytometric analysis of intracellular staining of HCV core antigen in infected HEPG2 cells

The intracellular staining of HCV core antigen in infected HepG2 cells was quantified by using a fluorescence activated cell sorting (FACS) based assay. Intracellular staining labeling was performed by direct immunofluorescence. HepG2 cells (collected after addition of trypsin) were centrifuged and supernatants were removed. Cell pellets were washed 4 times with PBS. For intracellular staining, cells were incubated with 4% paraformaldehyde for 10 min and 0.1% Triton X-100 in Tris buffer (pH 7.4) for 6 min. After washed with PBS, cells were incubated with FITC-labeled F(ab)₂ portion of HCV core antibody (at 1:2000 dilution) for 30 min at 4°C. Cells were washed with PBS containing 1% normal goat serum and suspended in 500 µL and analyzed by flow cytometry (FACS Calibure, BD). Mean fluorescence intensity was determined using Cell Quest software (Becton Dickinson).

Flow cytometric analysis of labeled E1 antigen on surface of infected HEPG2 cells

The surface staining of HCV E1 antigen in infected HepG2 cells was quantified by using a fluorescence activated cell sorting (FACS) based assay. Surface labeling was performed by direct immunofluorescence. HepG2 cells collected after trypsinization were centrifuged and supernatants were removed. Cell pellets were washed 4 times with PBS. Cells were incubated with FITC labeled HCV E1 antibody (at 1:1500 dilution) for 30 min at 4°C. Cells were washed 3 times with PBS containing 10 mL/L normal goat serum and suspended in 500 µL PBS and analyzed with flow cytometry (FACS Calibure, BD). Mean fluorescence intensity was determined using Cell Quest software (Becton Dickinson).

Western blot analysis of HCV antigens in HEPG2 cells

Uninfected and infected HepG2 cell lysates were subjected to sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE)^[17] through 40 g/L stacking and 160 g/L resolving gels in 0.75 mm-thick vertical slab gels. Cell lysate samples were diluted at 1:25 in PBS, mixed with the sample buffer (0.125 mol/L Tris base, 40 g/L SDS, 2% glycerol, 100 g/L mercaptoethanol, and 1 g/L bromophenol blue as a tracking dye) and immediately boiled for three min. A mixture of reference proteins was run in parallel. Gels were then stained with Coomassie blue. Western blotting was performed as follows: resolved samples separated by SDS-PAGE were electro-transferred onto nitrocellulose membranes (0.45 mm pore size). On the next day, membranes were cut into individual strips each of 0.3 mm width. Strips were washed 3 times with PBS-3 g/L T each for 5 min and blocked against non specific binding at room temperature for 1 h in PBS-3 g/L T-10 g/L bovine serum albumin (BSA). Strips were washed 3 times as above and incubated with diluted first antibody (infected human serum at 1:100, or anti-core/envelope rabbit antibodies at 1:500) in PBS-3 g/L T at room temperature for 2 h. After washed 3 times, strips were incubated with diluted peroxidase-labeled second antibodies (anti-human IgG/IgM mixture at 1:5000 in PBS-3 g/L T for previously treated strips

with human sera or anti-rabbit IgG at 1:1000 in PBS-3 g/L T for those treated with rabbit anti-core/envelope antisera. Both antibodies were from Jakson Immuno Research Laboratories; Dianova, Hamburg, Germany) for 2 h at room temperature. Visualization of immune complexes on the nitrocellulose membrane was done by developing the strips with 0.01 mol/L PBS (pH 7.4) containing 50 mg diaminobenzidine (Sigma; Deisenhofen, Germany) and 100 µL of 30 mL/L hydrogen peroxide.

Isolation and extraction of RNA from serum and HEPG2 cells

Isolation and extraction of RNA from serum and HEPG2 cells were performed as reported in our previous study^[18]. Briefly, cells were precipitated and washed in the same buffer to remove adherent viral particles before lysis in 4 mol/L guanidinium isothiocyanate containing 25 mmol/L sodium citrate and 0.5% sarcosyl and 0.1 mol/L b-mercaptoethanol. Cellular RNA was extracted using the single-step method described originally by Chomczynski and Sacchi^[19].

PCR of genomic RNA strands of HCV

Reverse transcription-nested PCR was carried out according to Lohr *et al*^[20] with few modifications. Retrotranscription was performed in 25 mL reaction mixture containing 20 units of AMV reverse transcriptase (Clontech, USA) with either 400 ng of total PBMC RNA or 3 mL of purified RNA from serum samples (equivalent to 30 mL serum) as template, 40 units of RNasin (Clontech, USA), a final concentration of 0.2 mmol/L from each dNTP (Promega, Madison, WI, USA) and 50 pmol of the reverse primer P1 (for sense strand) or 50 pmol of the forward primer P2 (for anti-sense strand). The reaction was incubated at 42°C for 60 min. and denatured at 98°C for 10 min. Amplification of the highly conserved 5'-UTR sequences was done using two rounds of PCR with 2 pairs of nested primers. The first round amplification was done in 50 mL reaction containing 50 pmol from each of P2 forward primers and P3 reverse primers, 0.2 mmol/L from each dNTP, 10 µL from RT reaction mixture as template and 2 units of Taq DNA polymerase (Promega, USA) in 1 × buffer supplied with the enzyme. The thermal cycling protocol was as follows: 1 min at 94°C, 1 min at 55°C and 1 min at 72°C for 30 cycles. The second round amplification was done as the first round, except for use of the nested reverse primer P4 and forward primer P5 at 50 pmol each. A fragment of 172 bp was identified in positive samples. Primer sequences are as follows: P1: 5' ggtgcacggctcagagacctc 3'; P2: 5' aactactgtcttcacgcagaa 3'; P3: 5' tgctcatgggtgcacggctc 3'; P4: 5' actcggtagcagctcgcg 3'; P5: 5' gtcgacctcaggacc 3'. To control false detection of negative-strand HCV RNA and known variations in PCR efficiency^[21,22], specific control assays and rigorous standardization of the reaction were employed as previously described^[20]. These specific control assays were cDNA synthesis without RNA templates to exclude product contamination, cDNA synthesis without RTase to exclude Taq polymerase RTase activity, cDNA synthesis and PCR step done with only the reverse or forward

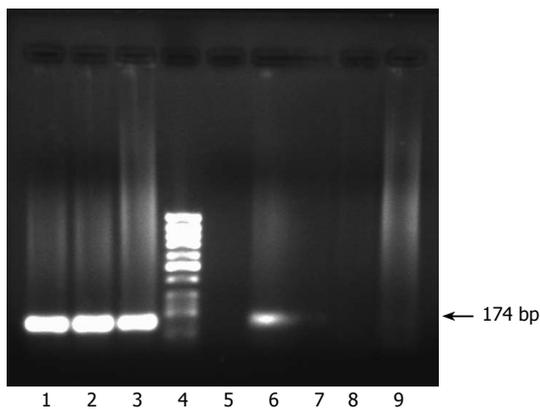


Figure 1 Establishment of an *in vitro* infection experiment of HepG2 cells with serum of a HCV genotype 4-infected patient and monitoring success of infection by nested RT-PCR amplification of viral sense and minus strands. The patient was confirmed to be infected with HCV as demonstrated by nested RT-PCR amplification of viral positive strand in the serum (lane 1), and both viral positive and negative strands (lane 2 and lane 3 respectively) in the peripheral blood mononuclear cells. After infection of the cells with the patient sera (see the detailed method in the materials and methods), cells were carefully washed and nested RT-PCR was carried out on the last wash to make sure that the culture medium contained no more viral RNA and results showed no amplified products (lane 5). At this stage we were sure that any detection of viral RNA within the cells could reflect successful viral adsorption and penetration. Three days after infection, RNA was extracted from both cells and their supernatant and nested RT-PCR for detection of both viral strands was carried out and results showed the presence of the sense strand in the cells (lane 6) but not in the supernatant (lane 7). The antisense strand was neither present in the cells nor in the supernatant (lanes 8, 9). Lane 4 is molecular weight standard DNA marker (ϕ -X-174/HaeIII; Q-BIOgene, Germany).

primer to confirm no contamination from mixed primers. These controls were consistently negative. In addition, cDNA synthesis was carried out using only one primer followed by heat inactivation of RTase activity at 95°C for 1 h, in an attempt to diminish false detection of negative-strand prior to the addition of the second primer.

RESULTS

Establishment of HCV HEPG2 cells in culture

Success of infection was monitored by nested RT-PCR amplification of viral sense and antisense (minus) strands (Figure 1). To confirm the infection of HCV in a patient with chronic active hepatitis whose serum was used in infection of HepG2 cells, nested RT-PCR amplification of viral sense strand in the serum (lane 1) and both viral sense and antisense strands (lane 2 and lane 3 respectively) in peripheral blood mononuclear cells were demonstrated. The viral load was quantified in patient's serum as 2.9×10^5 using real time PCR method (results not shown). After infection of the cells with the patient's sera, cells were carefully washed and nested RT-PCR was carried out on the last wash to make sure that the cell wash contained no more viral RNA (lane 5). At this stage we were sure that any detection of viral RNA within the cells could reflect successful viral binding and entry. Three days after infection, RNA was extracted from cells and culture media. Nested RT-PCR was carried out for detection of both viral strands. Results shown in Figure 1 displayed the presence of sense RNA strand in the cells (lane 6) but

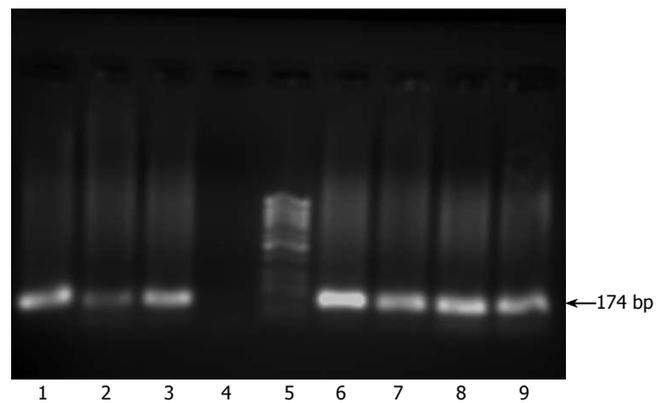


Figure 2 Monitoring of active viral replication at regular time intervals post infection of HepG2 cells. RNA was extracted from infected cells and infectious supernatants and their passages at 1, 2 and 4 wk post infection and nested RT-PCR was carried out for detection of both viral strands. Results showed the presence of both viral strands in RNA extracted from cells 1 wk post infection (lanes 1, 2) but only the sense strand was detectable in the supernatant at this time point (lane 3) while the antisense strand was absent (lane 4). At 2 and 4 wk post infection, both viral strands were detectable in both cells and supernatant. Lanes 6-9 show the presence of both sense and antisense strands in both cells and supernatant at 4 wk post infection. Lane 5 shows molecular weight standard DNA marker (ϕ -X-174/Hae III; Q-BIOgene, Germany).

not in the culture media (lane 7). The negative strand was neither present in the cells nor in the culture media (lanes 8, 9).

Monitoring of active viral replication at regular time intervals after infection of HEPG2 cells

RNA was extracted from infected cells and infectious supernatants and their passages at 1, 2 and 4 wk post infection and nested RT-PCR was carried out for detection of both viral strands (Figure 2). Results showed the presence of both viral strands in RNA extracted from cells 1 wk post infection (lanes 1, 2) but only the positive strand was detectable in the supernatant at this time point (lane 3) while the negative strand was undetectable (lane 4). At 2 and 4 wk post infection, both viral strands were detectable in both cells and supernatant. Lanes 6-9 show the presence of both sense and antisense strands in both cells and supernatants 4 wk post infection. Results at 2 wk were not demonstrated.

Monitoring infection of HEPG2 cells using culture medium from primary infected cells by nested RT-PCR

After incubation of HepG2 cells with infectious medium presumably containing exocytosed viral particles from primary infected cells, *de novo* infected cells were carefully washed to get rid of any viral traces and the last wash was checked for presence of viral RNA using nested RT-PCR which produced no amplified products (Figure 3, lane 1). RNA was extracted from infected cells as well as their culture media at 3 d, 1 wk, 2 wk and 4 wk post co-incubation with the infectious medium and subjected to nested RT-PCR to check the presence of either or both viral strands (Figure 3). After 3 d the cells contained only sense viral strand (lane 2) while the anti-sense strand was undetectable (lane 3). The supernatant contained neither strand (lanes 4, 5). After 1 wk post infection,

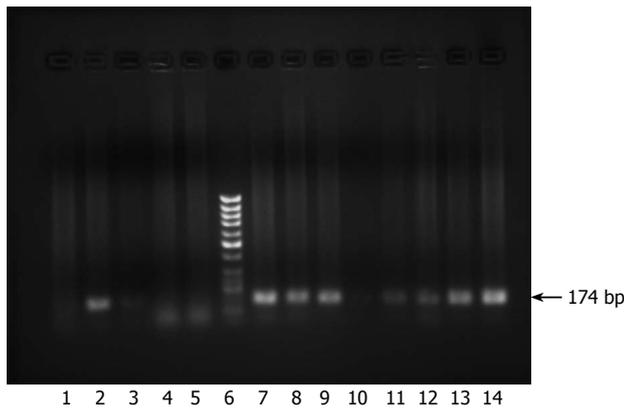


Figure 3 Monitoring infection of HepG2 cells using culture medium from primary infected cells by nested RT-PCR. After incubation of HepG2 cells with infectious medium presumably containing exocytosed virions from primary infected cells, cells were carefully washed to get rid of any viral traces and the last wash was subjected to RNA extraction followed by nested RT-PCR. Results showed no amplification of positive strand products (lane 1). RNA was extracted from infected cells and supernatants at 3 d, 1, 2 and 4 wk post co-incubation with the infectious supernatant and subjected to nested RT-PCR to check for the presence of each viral strand. At 3 d the cells contained only the positive viral strand (lane 2) but the negative strand was undetectable (lane 3). However, the supernatants contained none of the strands, only primers used could be seen (lanes 4, 5). Lane 6 is molecular weight standard DNA marker (ϕ -X-174/HaeIII; Q-BIOgene, Germany). At 1 wk post infection, both sense and antisense strands were detectable in the RNA extracted from the infected cells (lanes 7, 8), whereas the supernatant contained only the sense strand (lane 9) but not the antisense strand (lane 10). At 2 and 4 wk, RNA extracted from infected cells as well as the supernatants contained both positive and negative strands. Results of amplification for both positive and negative strands for cellular RNA are shown (2 wk, lanes 11, 12) and (4 wk, lanes 13, 14).

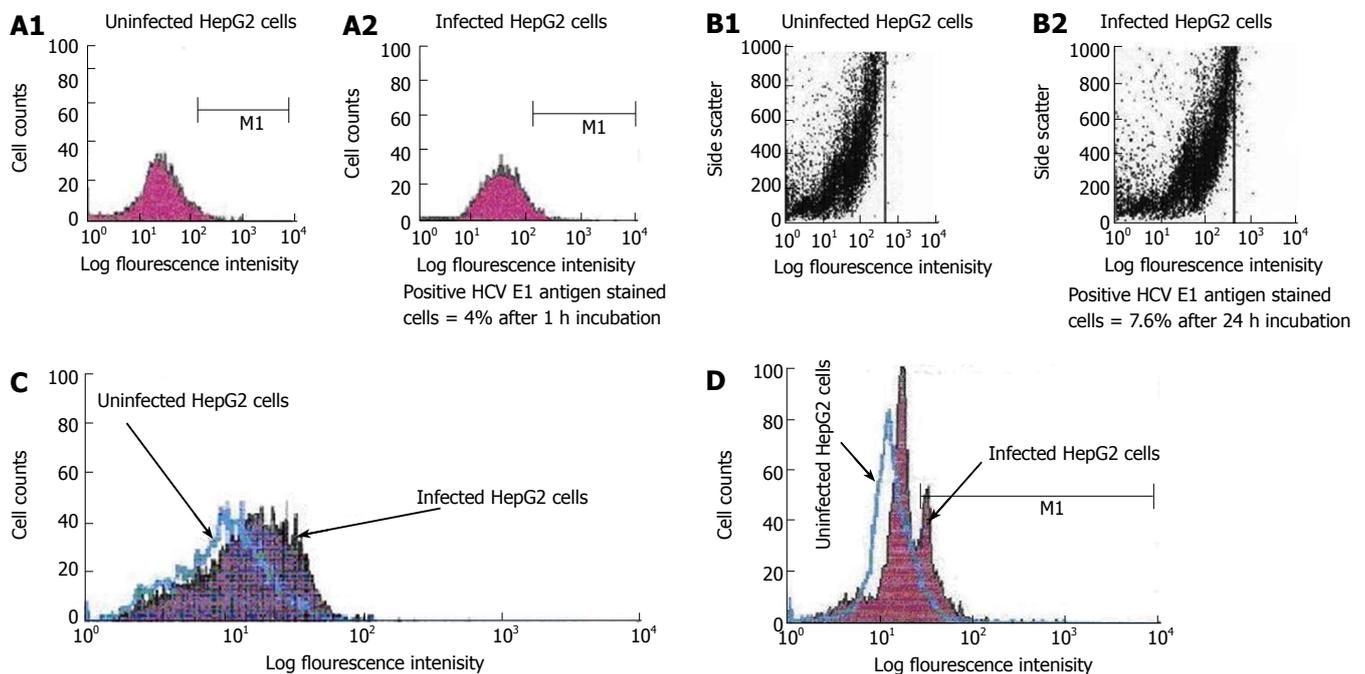


Figure 4 **A:** Single parameter histogram for flow cytometric analysis of surface staining of HCV E1 gene expression on the infected HepG2 cells after one hour incubation. HepG2 cells were incubated with PBS (**A1**) (uninfected) or with HCV positive serum (**A2**) (infected) for 1 h incubation. Cells were harvested and stained with FITIC labeled HCV anti-E1 antibody as described in materials and methods; **B:** Dot histogram for flow cytometric analysis of surface staining of HCV E1 gene expression on the infected HepG2 cells after 24 h incubation. HepG2 cells were incubated with PBS (**B1**) (uninfected) or with HCV positive serum (**B2**) (infected) for 24 h incubation. Cells were harvested and stained with FITIC labeled HCV anti-E1 antibody; **C:** Overlap histogram for flow cytometric analysis of surface staining of HCV E1 gene expression on the infected HepG2 cells after one week incubation. HepG2 cells were incubated with PBS (uninfected) or with HCV positive serum (infected) for one week incubation. Cells were harvested and stained with FITIC labeled HCV anti-E1 antibody; **D:** Overlap histogram for flow cytometric analysis of intracellular staining of HCV core gene expression in the infected HepG2 cells after 3 d incubation. HepG2 cells were incubated with PBS (uninfected) or with HCV positive serum (infected) for 3 d incubation. Cells were harvested and stained with FITIC labeled HCV anti-core antibody. Labeled cells were analyzed with flow cytometry (FACS Calibure, Becton Dickinson).

both sense and antisense viral strands were detectable in infected cells (lanes 7, 8), whereas the supernatant contained only the sense strand (lane 9) while the antisense strand was undetectable (lane 10). After 2 and 4 wk post infection, RNA extracted from infected cells as well as their supernatants contained both positive and negative strands. Results of amplification of both positive and negative strands from cellular RNA at 2 and 4 wk are presented in lanes 11-14. Results of the nested RT-PCR on the supernatant at the same time points were not demonstrated since they were identical to those obtained from the cells. However, culture supernatant from infected HepG2 cells was used to infect naïve (uninfected) cultured

HepG2 cells and we found that these HepG2 cells were infected as detected by RT-PCR (Data not shown).

Flow cytometric analysis of surface and intracellular staining of HCV antigen expression in infected HEPG2 cells

Flow cytometric analysis showed that HCV core and E1 antigens were detected on surface and inside of the infected HepG2 cells. Figures 4A-C show the percentage of anti E1 positive staining on the surface of HepG2 cells after 1 h (4%) and 24 h (7.6%) and one week (12.5%) of incubation of HepG2 cells with positive HCV serum sample. Core protein was detectable in 5.7% of cells after

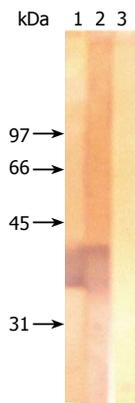


Figure 5 Testing translation of viral E1 in supernatant and lysates of HepG2 cells infected with HCV from 1 mo culture by Western blot analysis. Supernatant (strip 1) and lysates (strip 2) of HepG2 cells infected with HCV were subjected to Western blot analysis, hybridization with the anti-E1 antibody clearly showed the expression of a cluster of immunogenic proteins at molecular weights localized between 31 and 45 kDa. This cluster was undetectable on the strip immobilized with uninfected HepG2 cell lysates (strip 3).

24 h, and increased to 13.5% of cells after 3 d. Figure 4D shows the intracellular staining of core antigen using F(ab)₂ portion of the core antibody after infection of 3 d.

Western blot analysis of HCV viral antigen expression in infected HEPG2 cell lysates

When supernatant (Figure 5, strip 1) and lysates (strip 2) of HepG2 cells infected with HCV were subjected to Western blot analysis, hybridization with the anti-E1 antibody could clearly show the expression of a cluster of immunogenic peptides at molecular weights extended between 31 kDa and 45 kDa over 1 mo period. This cluster was undetectable on the strip immobilized with uninfected HepG2 cell lysates (strip 3).

DISCUSSION

Although knowledge of the molecular biology of HCV has progressed rapidly, our understanding of viral replication and pathogenicity is still hampered by the lack of reliable and efficient cell culture systems. To achieve a reliable *in vitro* system we need to obtain a biological status wherein viral-host interactions mimic exactly what happens naturally *in vivo*, since both viral and host factors make up together the overall outcome of the pathogenetic pathways. The reasons for using HepG2 cells in the current study include the great similarity in biosynthetic pathways between primary hepatocytes and HepG2 cells^[23]. Also the later cells contain LDL and CD81 receptors which are known to mediate HCV entry into cells^[24]. Validity of HepG2 cells in propagating HCV has been reported by other laboratories^[25]. The viral component of the model has several alternative strategies. Subgenomic or genomic replicons have been used in elucidating the replicative machinery of the virus^[26] but could not mimic the actual viral replication cycle and shedding of the virus to the culture medium. Despite the extremely robust *in vivo* replication rate of HCV using genomic replicons, efforts to propagate the virus in cell culture have been frustratingly unsuccessful^[27]. Thus the viral replication but not the biologically relevant infectious viral particles can be demonstrated by such approach. In the present study we utilized infectious serum with native viral particles presumably containing the full length viral RNA genome in infecting HepG2 cells *in vitro*. The recent understanding

of the HCV molecular biology demonstrates that both 5' and 3' untranslated regions of the viral RNA genome play a pivotal role in translation of viral proteins *via* interaction with cellular factors including eukaryotic initiation factor 3 eIF3^[35], 40S ribosomal subunit^[28] and poly pyrimidine tract binding (PTB)^[29] protein. Besides, it has been shown that intra genetic viral interactions such as NS4a/NS5a are required for key pathways in HCV life cycle. In the current study, the use of infectious viral particles containing intact RNA genome could guarantee the presence of the necessary elements involved in translation of polyprotein precursor and viral replication. We have presented several lines of evidence that the cell model described herein maintains HCV life cycle. A minor fraction of cells (4%) had a detectable viral envelope on cell surface as early as one hour after incubation. This fraction steadily increased to 7.6% in 24 h and 12.5% after one week. E1 protein reached detectable levels by Western blotting analyses at both intracellular and extra cellular compartments after one month. *De novo* synthesis of RNA minus strand was detected inside HepG2 cells as early as one week post infection and appeared in the medium one week later. However, the detection of the replicative intermediate (antisense strand HCV RNA) is thought to be reasonable for assessment of HCV replication. Because detectable HCV structural proteins in cells after infection may represent the residue of the inoculated virus after releasing the viral genome to cytoplasm, it is necessary to demonstrate that HCV structural proteins detected in the infected cultures are newly synthesized rather than residuals of viral inoculum. Interestingly, the core protein was only detectable in 5.7% of cells after 24 h and increased to 13.5% of cells after 3 d, indicating that such observed increase in core expression reflects part of *de novo* synthesized structural viral proteins. The ability of culture medium to transmit viral particles to new cells later in one month culture with concomitant detection of core (results not shown) and envelope proteins as well as detection of sense and antisense RNA strands suggest that infected HepG2 cells reach a state of equilibrium after one month of infection. Other cellular models for HCV propagation can transmit viral particles to naïve cells^[30]. We assume that this approach brings our *in vitro* system to become closer to native viral infection status occurring *in vivo*. The expression of different viral antigens agrees with the earlier reports that liver and blood cells from infected patients do support these expressions^[11,31-35]. Our observation that HCV RNA detection was intermittent during early days post infection agrees with previous reports on infection experiments^[7,10,33], a finding which has led the investigators to suspect the consistency of viral replication and gene expression in these cell models.

In conclusion, we report an *in vitro* system of cultured HepG2 cells infected with HCV particles. These cells support viral replication and gene expression. The consistent expression of viral proteins and the ability of culture medium to transmit the virus to new cells make this model optimum for studying HCV life cycle, screening for anti HCV drugs and testing the efficacy of therapeutic antibodies.

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Expression of tissue factor in pancreatic adenocarcinoma is associated with activation of coagulation

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were significantly elevated compared to controls whereas elevated F1 + 2 levels did not reach statistical significance compared to controls. In CP patients TAT and F1 + 2 levels proved to be significantly elevated compared to controls, although TAT elevation was less pronounced than in PCa patients.

CONCLUSION: We conclude that in addition to the upregulated expression of TF on the cell membrane, soluble TF might contribute to activation of the coagulation system in pancreatic cancer.

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Key words: Coagulation activation; Pancreatic carcinoma; Thromboembolism; Thrombosis; Tissue factor

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Abstract

AIM: To study expression of tissue factor (TF) in pancreatic cancer and its role in the development of thromboembolism.

METHODS: TF expression was studied in eight human pancreatic carcinoma cell lines by Northern blot and indirect immunofluorescence. Expression of alternatively spliced TF (asTF) was assessed by RT-PCR. In addition, TF expression was determined by immunofluorescence in pancreatic tissues of 19 patients with pancreatic adenocarcinoma (PCa), 9 patients with chronic pancreatitis (CP) and 20 normal controls. Plasma samples (30 PCa-patients, 13 CP-patients and 20 controls) were investigated for soluble TF levels and coagulation activation markers [thrombin-antithrombin III complex (TAT), prothrombin fragment 1 + 2 (F1 + 2)].

RESULTS: All pancreatic carcinoma cell lines expressed TF (8/8) and most of them expressed asTF (6/8). TF expression at the protein level did not correlate with the differentiation of the carcinoma cell line. All but two pancreatic cancer tissue samples stained positive for TF (17/19). In all samples of CP weak staining was restricted to pancreatic duct cells, whereas only a few subendothelial cells were positive in 9/20 of normal controls. TF and TAT levels in PCa patients

INTRODUCTION

Cancer patients are highly susceptible to thromboembolic complications. These thromboembolic complications include venous and arterial thrombosis, migratory thrombophlebitis, pulmonary embolism and disseminated intravascular coagulation (DIC). Idiopathic deep vein thrombosis may be the first clinical manifestation of an occult malignancy and the cancer risk is particularly increased within the first twelve months after the diagnosis of thromboembolism^[1].

Patients with idiopathic venous thrombosis have been recognized to have a three to four fold increased likelihood of harboring malignancies^[2,3]. Cancer patients have a two to eight fold higher risk of dying after an acute thrombotic event than patients without cancer^[4-6]. Furthermore, pancreatic cancer is associated with the highest risk for thromboembolism with an estimated risk ratio of 10^[7].

Although the first description of an association of malignant disease with thrombotic events dated back as early as 1865, the underlying mechanisms are poorly understood^[8].

Tissue factor (TF) is a key element in the initiation of the extrinsic coagulation cascade. Binding of activated factor VII to TF results in the activation of factor IX and X ultimately leading to thrombin formation which generates fibrin from fibrinogen and activates platelets^[9]. TF is a 49-kD transmembrane glycoprotein belonging to the cytokine receptor family group 2 and contains 263 amino acid residues. Procoagulant activity of TF is tightly regulated at the transcriptional level and the natural inhibitor tissue factor pathway inhibitor (TFPI) can inhibit uncontrolled activation of coagulation. Recently, a variant of TF was identified which results from alternative splicing of the primary RNA transcript. Alternatively spliced TF (asTF) proves to be biologically active^[10,11].

Constitutive TF expression is demonstrated predominantly in the brain, lung, placenta, cerebral cortex and kidney^[12,13]. Aberrant expression of TF, the principal initiator of blood coagulation, has been postulated to contribute to thrombosis in cancer patients.

Various tumor entities are shown to express TF, including glioma^[14], breast cancer^[15,16], non-small cell lung cancer^[17,18] and pancreatic cancer^[19,20].

The present study was designed to systematically investigate the expression of TF by pancreatic carcinoma cells *in vitro* and *in vivo*. Specifically, we focused on the following questions: Do ductal pancreatic adenocarcinoma cells express TF and is the expression intensity related to the degree of tumor differentiation? Is the expression of TF restricted to pancreatic carcinoma cells? Does expression of TF adversely affect blood coagulation in patients as analysed by coagulation activation markers? Is clinical thromboembolism in these patients related to plasma coagulation activation?

MATERIALS AND METHODS

Cell lines

Eight human pancreatic carcinoma cell lines were studied for TF expression: AsPC-1, BxPC-3, Capan-1, Capan-2, PaCa-2, PaCa-3, PaCa-44, and PANC-1. The cell lines have been previously studied and characterized^[21-23]. The grading of the tumor cell lines as assessed by electron microscopy^[21] is listed in Table 1. The expression of asTF was assessed in eight pancreatic cancer cell lines (AsPC-1, BxPC-3, Capan-1, Capan-2, PaCa-44, PANC-1, NP9, NP29), in two colorectal carcinoma cell lines (DLD-1 and SW48), in the cervical cancer cell line HeLa and in fibroblasts.

Cells were grown in their respective medium (usually RPMI-1640) supplemented with 10% fetal calf serum. For serum deprivation, cells were seeded in tissue culture flasks and incubated with medium containing 10% fetal calf serum for 24 h, then washed twice with sterile PBS, and finally incubated with serum-free medium.

Northern blot

RNA was extracted from exponentially growing cell lines as described earlier^[22]. Twenty μ g of total RNA was analysed by formamide agarose gel electrophoresis and Northern blotting^[22]. Blots were hybridized to a TF cDNA

Table 1 Tissue factor expression in eight human pancreatic adenocarcinoma cell lines in relation to the histological differentiation determined by northern-blot analysis (RNA level) and by indirect immunofluorescence (protein level)

	Grade	RNA	Protein
Capan-1	I	1 ¹	2
Capan-2	I - II	3	2
AsPC-1	I - II	3	3
BxPC-3	II	3	2-3
PANC-1	II - III	1	1-2
PaCa-2	II - III	1	2-3
PaCa-3	III	1 ¹	3
PaCa-4	III	1	1-2

¹Positive only in the absence of fetal calf serum.

Table 2 Overview of oligonucleotides used for amplification of TF and asTF

Type of primer	Oligonucleotide
Forward	5'-CAGGCACTACAAATACTGTGGCAG-3'
Reverse	5'-TGCAGTAGCTCCAACAGTGCTCC-3'

TF: Tissue factor; asTF: Alternatively spliced TF.

probe^[24] and subsequently to a ribosomal cDNA control probe (S138). The 1.4 kb TF-specific insert was labelled with 32P-dATP yielding $5-7 \times 10^8$ cpm per μ g DNA. Filters were hybridized with $1-2 \times 10^6$ cpm/mL hybridization mix at 42°C overnight, washed with $2 \times$ SSC at 37°C for 30 min and $0.5 \times$ SSC at 55°C for 10 min, dried, and exposed to Kodak XAR film using Kronex lightning enhancer screens. Films were evaluated semiquantitatively according to an established rating. A minimal or no signal representing densitometric measurements < 0.100 was rated 0; a weak signal equivalent to densitometric values 0.100 to 1.0 was rated 1; a signal equivalent to densitometric values 1.0 to 5.0 was rated 2, and a signal equivalent to densitometric readings > 5.0 was rated 3^[21,22].

RT-PCR

For detection of concomitant expression of TF and asTF, cells were washed with PBS. After complete removal of the PBS, 350 μ L buffer RLT (RNeasy Mini Kit; Qiagen, Hilden, Germany) plus β -ME (10 μ L/mL) was pipetted directly onto the cells. The lysate was processed further according to the manufacturer's recommendations. Isolated total RNA (1 μ g, determined photometrically) was reverse transcribed using Superscript II reverse transcriptase (Invitrogen; Karlsruhe, Germany) and oligo-dT primers. The resulting cDNA was amplified in separate tubes using Hot Star Taq (Qiagen; Hilden, Germany). Used primers are listed in Table 2. Oligonucleotides corresponded to TF nucleotides 221-244 and 1013-1036, respectively. TF and asTF were discriminated on a 2% agarose gel as bands of 815 and 656 bp, respectively. The cycling parameters are as follows: initial denaturation and Taq polymerase activation at 94°C for 15 min, cycling (33 \times) at 94°C for 45 s, 50°C for 45 s and 72°C for 45 s and a final extension at 72°C for 5 min.

Immunofluorescence

Tumor cell lines grown on glass slides, cryostat sections of pancreatic carcinoma, chronic pancreatitis, and of normal pancreatic tissue were fixed in acetone at -20°C for 10 min and immunostained by indirect immunofluorescence. The primary mouse monoclonal antibody against TF was clone 5G9^[25]. Staining was performed on at least two separate preparations for all tissue sections or cell lines. Staining intensity was graded semiquantitatively as described before^[21,22]: a negative staining was rated 0, weakly positive 1, moderately positive 2, and strongly positive 3. This referred to the overall staining intensity. If there were focal positive spots, it was documented separately. Appropriate controls were included throughout this investigation.

Pancreatic tissue

Forty-eight tissue samples were available for further investigation: 19/30 samples from patients with pancreatic carcinoma and 9/13 samples from patients with chronic pancreatitis. Twenty normal tissue samples were obtained from organ donor pancreata^[26]. After surgical removal the tissue sample was immediately snap frozen in liquid nitrogen. Five micrometer thick sections were stained with hematoxylin and eosin for histological examination^[27].

Patients

The study population comprised 30 patients with pancreatic ductal adenocarcinoma (PCa). Thirteen patients with chronic pancreatitis (CP) and 30 healthy subjects (co) served as controls. Clinically overt thromboembolic events were noted in four patients.

With patients' written informed consent citrate-anticoagulated blood was drawn on the first day of hospitalization prior to the administration of medications potentially interfering with the coagulation system. Platelet-free plasma aliquots were stored at -80°C .

All patients underwent extensive diagnostic work-up including abdominal ultrasound, contrast-enhanced computed tomography and magnetic-resonance tomography, respectively. Suspected thromboembolic manifestations were investigated by doppler ultrasound and when necessary with computed tomography and angiography.

Coagulation studies

Separate plasma aliquots obtained from patients were quickly thawed and utilized for the following commercially available assays according to the instructions provided by the manufacturers. TF antigen concentration was measured using the Imubind[®] Tissue Factor ELISA kit (American Diagnostics, Greenwich, CT, USA). Thrombin-anti-thrombin III complex (TAT) was quantitated employing the Enzygnost[®] TATmicro enzyme immunoassay (Behring, Frankfurt, Germany). Prothrombin fragment 1 + 2 (F1 + 2) was evaluated using the Enzygnost[®] F1 + 2 kit (Behring, Frankfurt, Germany).

Statistical analysis

Plasma levels of TF, F1 + 2 and TAT are expressed as the means \pm SD. Statistical significance was calculated using the two-sided Mann-Whitney test. A *P*-value of < 0.05 was considered statistically significant.

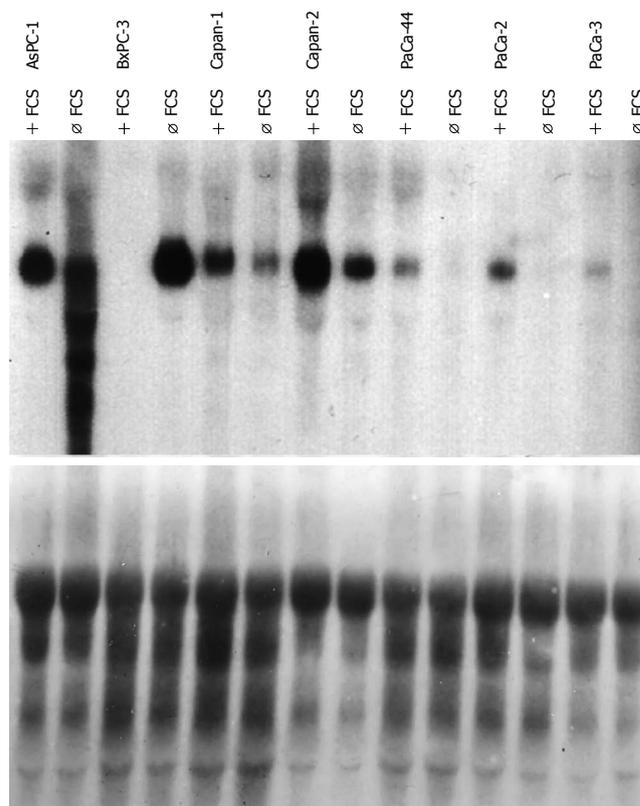


Figure 1 Northern blot of human pancreatic carcinoma cell lines utilizing a cDNA probe for tissue factor (TF) and a ribosomal cDNA control probe (S138). Cell culture conditions with and without (\emptyset) fetal calf serum (FCS).

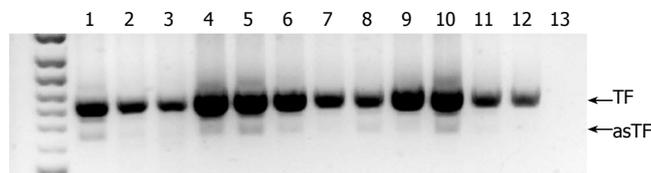


Figure 2 Assessment of TF and asTF expression by RT-PCR. 1: DLD-1; 2: SW48; 3: PANC-1; 4: BxPC-3; 5: PaCa-44; 6: Capan-2; 7: Capan-1; 8: AsPC-1; 9: NP9; 10: NP29; 11: HeLa; 12: Fibroblasts; 13: Negative control.

RESULTS

Expression of TF in pancreatic carcinoma cell lines

All of the eight human pancreatic carcinoma cell lines expressed TF (Table 1, Figures 1, 2, 3A and B). Expression at RNA-level could be modulated in part by serum depletion (Figure 1). All lowly differentiated cell lines (grade II and II-III) exhibited weak TF expression determined by Northern-blot analysis, whereas three of the four highly differentiated cell lines (grade I, I-II and II) were found to have a strong TF expression at the RNA level. In contrast, no clear correlation between differentiation and TF protein levels could be demonstrated by immunofluorescence (Table 1).

Expression of asTF in pancreatic cancer cell lines

In addition to TF, the majority of studied pancreatic carcinoma cell lines (6/8) expressed asTF (Figure 2). Furthermore, asTF expression could be demonstrated in the colorectal carcinoma cell lines DLD-1. The colorectal

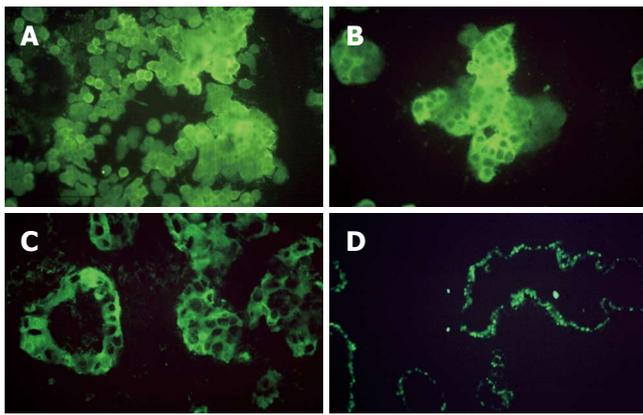


Figure 3 Representative demonstration of tissue factor (TF) expression by immunofluorescence in AsPC-1 (A) and CAPAN-1 (B) pancreatic cancer cell lines, in tissue of pancreatic cancer (C) and chronic pancreatitis (D).

Table 3 Immunofluorescence staining analysis of tissue factor expression in patients with pancreatic carcinoma (PCa), chronic pancreatitis (CP) and in healthy controls (Co)

	PCa (n = 19)	CP (n = 9)	Co (n = 20)
Total immunoreactivity	17 (84%) ¹	9/9 (100%) ²	9/20 (45%) ³
Grade 3	4	0	0
Grade 2	7	0	0
Grade 1	6	9*	0
Negative	2	0	11

¹P = 0.027 vs controls; ²A few positively stained epithelial duct cells; ³Expression restricted to subendothelial cells.

carcinoma cell line SW48, the cervical cancer cell line HeLa and fibroblasts did not express asTF.

Expression of TF in pancreatic tissue

Of the 19 pancreatic carcinoma tissue samples nearly all (17/19) were positive for TF (4/19: grade 3, 7/19: grade 2, 6/19: grade 1) and only two were negative (Table 3, Figure 3C). The amount of TF expression in tumor tissue did not correlate with the grading of the tumor (Table 3).

All tissue samples obtained from chronic pancreatitis demonstrated positive staining of epithelial pancreatic duct cells (Table 3, Figure 3D). However, staining intensity was rated weakly in these cases. Nearly half of all normal control tissue samples exhibited only weak staining of a few subendothelial cells (9/20) (Table 3).

Plasma concentrations of TF in patients with pancreatic cancer and healthy controls

TF plasma concentrations in 30 patients with pancreatic cancer were significantly enhanced versus 30 healthy controls (288 ± 41.4 ng/L vs 139 ± 9.7 ng/L, P = 0.005) (Figure 4).

Coagulation studies

Plasma levels of TAT in patients with pancreatic carcinoma (PCa) were more than twenty-times higher than in normal controls (89.4 ± 38.8 µg/L vs 4.0 ± 3.5 µg/L, P = 0.002). Interestingly, they were also significantly enhanced

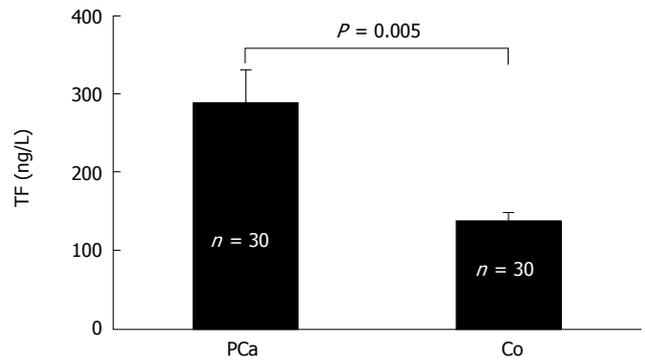


Figure 4 Plasma concentrations of tissue factor (TF) in patients with pancreatic cancer (PCa) versus controls (Co). Bars indicate means and standard deviation.

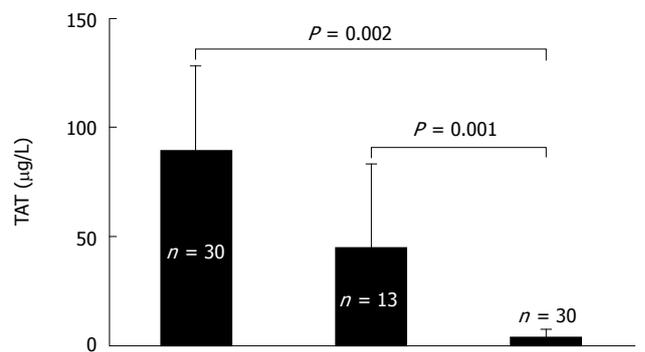


Figure 5 Plasma concentrations of thrombin-antithrombin complex (TAT) in patients with pancreatic cancer (PCa), chronic pancreatitis (CP) and healthy controls (Co). Bars indicate means and standard deviation.

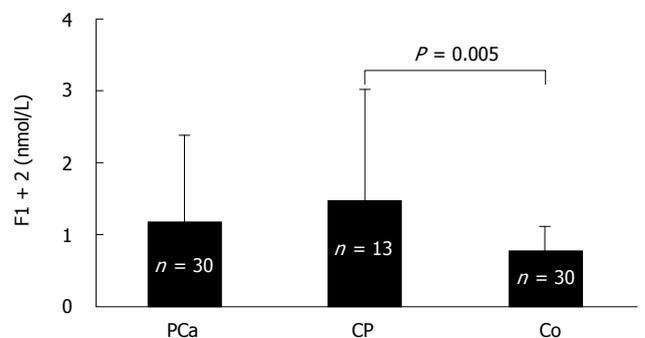


Figure 6 Plasma concentrations of prothrombin fragment 1 + 2 (F1 + 2) in patients with pancreatic cancer (PCa), chronic pancreatitis (CP) and healthy controls (Co). Bars indicate means and standard deviation.

in patients with chronic pancreatitis (CP) versus healthy controls (45.1 ± 37.9 µg/L vs 4.0 ± 3.5 µg/L, P = 0.001) (Figure 5). However, higher levels in PCa patients compared to CP patients did not reach statistical significance.

Plasma levels of prothrombin F1 + 2 proved to be higher in patients with pancreatic adenocarcinoma although not reaching statistical significance (1.16 ± 1.56 nmol/L vs 0.75 ± 0.34 nmol/L, P = 0.16), whereas significant elevation of F1 + 2 was observed in patients with chronic pancreatitis versus controls (1.46 ± 1.23 nmol/L vs 0.75 ± 0.34 nmol/L, P = 0.005) (Figure 6). There was no correlation between the plasma levels of

Table 4 Patients with pancreatic carcinoma complicated by clinically overt thromboembolism

Case	Sex/age	Stage ¹	Grade	TF ²	TF (pg/mL)	Location of thromboembolism
1	m/65	4	II	3	291	Splenic vein thrombosis
2	m/69	4	II	2	nd	Mesenteric artery thrombosis
3	f/61	4	II	1 ³	391	Upper jugular vein thrombosis
4	m/62	4	II	2	nd	Pulmonary embolism

¹ Staging according to UICC; ² Results from immunohistochemistry, i.e. immunofluorescence for TF; ³ TF was focally positive; nd: not determined.

TAT or F1 + 2 and the extent of TF expression in the pancreatic tumor tissue (data not shown). However, there was a strong correlation between TAT and F1 + 2 plasma levels ($P < 0.05$).

Patients with pancreatic cancer and thromboembolic complications

Four of the TF-positive patients had thrombosis, as revealed by doppler ultrasound, computed tomography and/or angiography (Table 4). All patients with thrombosis had a stage 4 pancreatic carcinoma. In two cases TF plasma levels were available which were increased by a factor of 2 and 2.8, respectively, compared to the mean value of TF plasma levels in healthy controls.

DISCUSSION

Our study proved expression of TF by epithelial tumor cells in pancreatic adenocarcinoma *in vitro* and *in vivo*. In patients with pancreatic cancer, a hypercoagulable state could be confirmed by increased concentrations of the TAT in conjunction with elevated TF plasma levels. Four patients expressing TF in their tumors developed clinically overt thromboembolism.

The presence of a malignant disease dramatically increases the risk for thromboembolic events. Immobility, indwelling catheters, surgical procedures and chemotherapy represent risk factors rendering cancer patients for thromboembolism^[28]. In addition, direct activation of the coagulation cascade by cancer cells is considered a key feature of the well-established increased risk for thrombotic events. TF plays a central role in activating coagulation and enhanced expression is implicated in diseases with enhanced thrombotic features like cancer^[29], atherosclerosis^[30], sickle cell disease^[31] and sepsis^[32].

We analyzed the expression of TF in eight well characterized cell lines of ductular adenocarcinoma^[23]. Without exception all showed TF expression at RNA and protein levels. Highest RNA levels were found in the well-differentiated cancer cell lines (Capan-2, AsPC-1, BxPC-3). However, in relation to differentiation no clear correlation of TF expression could be observed at the protein level. The fact that the amount of TF protein levels did not strictly correspond to RNA levels suggests that posttranscriptional regulation might be of importance in TF expression. We could not confirm the finding of Kakkar that expression of TF was most prominent in poorly differentiated tumors^[20].

In addition to expression of TF, the majority of studied pancreatic carcinoma cell lines expressed the recently characterized asTF^[10,11]. This splice variant contains the extracellular domain of TF, but lacks the transmembrane and cytoplasmic domain. Exon 4 is spliced directly to exon 6 leading to a frameshift so that the translated peptide comprises residues 1-166 of the extracellular domain of TF and a unique C terminus (residues 167-206). AsTF proved to have full pro-coagulant activity. The expression of asTF might contribute to systemic hypercoagulopathy in pancreatic cancer.

Corresponding to the TF expression in pancreatic carcinoma cell lines we demonstrated, in the majority of tissue specimens of patients with pancreatic cancer, TF expression in tumor cells, which showed a high variability in the expression rate determined by immunofluorescence. Recently, upregulated TF expression in colorectal cancer cells was ascribed to *K-ras* mutations^[33]. As mutations in the *K-ras* oncogene are an early event in the development of pancreatic cancer and as these mutations are found in the majority of pancreatic cancer patients, mutational activation of *K-ras* might have a contributory role in upregulated TF expression^[34]. Further studies are warranted to clarify whether inactivation of the tumor suppressor gene PTEN and hypoxia are involved in enhanced TF expression in pancreatic cancer as it was recently demonstrated in glioblastoma^[35]. Lately, Nitori and co-workers determined TF expression in pancreatic cancer by immunohistochemistry and found an association of high TF expression with metastasis and a low survival rate^[36].

To our knowledge this is the first report demonstrating an enhanced TF expression in tissues of patients with chronic pancreatitis which was confined to pancreatic duct cells. This was a surprising result as tissue of chronic pancreatitis patients was initially intended to be included in the study to serve as a negative control. Previous data have shown that the exposure of endotoxin, tumor necrosis factor (TNF)- α and interleukin (IL)-1 results in upregulation of TF expression *in vitro*^[37-40]. Proinflammatory cytokines secreted by the inflammatory infiltrate in chronic pancreatitis might be an explanation for the upregulated TF expression^[41]. In contrast, only weak staining of a few subendothelial cells was found in control tissue specimens. Upregulated TF expression in the tissue of chronic pancreatitis patients might have a contributory role in the development of thrombotic events, which is a well-known complication in chronic pancreatitis.

TF plasma concentrations in patients with pancreatic cancer were found to be more than twice as high than in normal controls which might stem from asTF or from TF-containing microparticles and from TF which is secreted by activated leukocytes, respectively^[42-44]. Further studies are needed to prove that the source of TF in the plasma of patients with pancreatic cancer is the tumor itself.

In parallel to enhanced TF expression, markers of thrombin formation (TAT, F1 + 2) were elevated in the plasma of patients with pancreatic cancer and chronic pancreatitis. A procoagulatory state in patients with chronic pancreatitis might facilitate the generation of portal and splenic vein thrombosis, which is commonly seen in this patients' group.

Four patients with pancreatic cancer had concurrent venous thrombosis or pulmonary embolism. TF expression was highly variable but all patients had a stage 4 carcinoma. Apparently the tumor load and tumor progression is a more powerful prognostic marker for thrombotic complications than TF expression in pancreatic cancer. This is in accordance with data showing that the stage of colorectal cancer most consistently correlates with the risk for thromboembolism^[45].

In this study we focused on TF expression, taking into account that undoubtedly multiple factors modulate hypercoagulable state.

Tissue factor pathway inhibitor (TFPI) is an important factor limiting the effects of TF. In a recent study low levels of TFPI were associated with an increased risk of venous thrombosis^[46]. Mice deficient of TFPI exhibited an increased rate of thrombosis and accelerated atherosclerosis^[47]. Both pancreatic carcinoma and chronic pancreatitis produce a rich stroma^[48]. We demonstrated that epithelial tumor cells can synthesize and deposit extracellular matrix proteins in pancreatic carcinoma^[21,49]. Fibronectin itself which is expressed by pancreatic tumor cells^[21] is capable of activating the coagulation cascade^[50].

In addition to its haemostatic potential, the TF expression by tumor cells has implications in tumor biology^[51,52]. Belting and coworkers have shown that TF directly promotes angiogenesis through protease-activated receptor-2 (PAR-2) signaling^[53]. Upregulation of the urokinase receptor in pancreatic cancer by TF enhances tumor invasion and metastasis^[54]. Pancreatic carcinoma cells possess a functioning thrombin receptor, and stimulation with thrombin representing the product of the coagulation cascade causes cell proliferation^[55]. Furthermore, thrombin has a proangiogenic function^[56,57].

In summary, enhanced TF expression in pancreatic adenocarcinoma in conjunction with elevated TF plasma levels might have a crucial role in hypercoagulopathy, resulting in thromboembolism.

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BASIC RESEARCH

DA-9601, a standardized extract of *Artemisia asiatica*, blocks TNF- α -induced IL-8 and CCL20 production by inhibiting p38 kinase and NF- κ B pathways in human gastric epithelial cells

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Abstract

AIM: To investigate whether, or how, DA-9601, which is a new gastroprotective agent, inhibits TNF- α -induced inflammatory signals in gastric epithelial AGS cells.

METHODS: Cell viability was determined by MTT assay. IL-8 and CCL20 promoter activities were determined by a luciferase reporter gene assay. NF- κ B-dependent transcriptional activity was determined by I- κ B α degradation, NF- κ B p65 nuclear translocation and a luciferase activity assay. IL-8 and CCL20 gene expression and protein secretion were determined by RT-PCR and an enzyme-linked immunosorbent assay (ELISA). Total and phosphorylated forms of mitogen-activated protein kinases (MAPKs) were determined by Western blot.

RESULTS: Treatment of AGS cells with DA-9601 reduced

TNF- α -induced IL-8 and CCL20 promoter activities, as well as their gene expression and protein release. TNF- α also induced NF- κ B-dependent transcriptional activity in AGS cells. In contrast, in cells treated with DA-9601, TNF- α -induced NF- κ B activity was significantly blocked. Although all three MAP kinase family members were phosphorylated in response to TNF- α , a selective inhibitor of p38 kinase SB203580 only could inhibit both NF- κ B-dependent transcriptional activity and IL-8 and CCL20 production, suggesting a potential link between p38 kinase and NF- κ B-dependent pathways in AGS cells. Interestingly, DA-9601 also selectively inhibited p38 kinase phosphorylation induced by TNF- α .

CONCLUSION: DA-9601 blocked TNF- α -mediated inflammatory signals by potentially modulating the p38 kinase pathway and/or a signal leading to NF- κ B-dependent pathways in gastric epithelial cells.

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Key words: CCL20; IL-8; *Artemisia asiatica*; DA-9601; TNF- α ; p38 kinase; NF- κ B

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INTRODUCTION

Artemisia asiatica has been frequently used in traditional Asian medicine for the treatment of diseases such as inflammation, cancer and microbial infection. Along this line, a novel antipeptic formulation prepared from the ethanol extracts of *A. asiatica*, namely DA-9601 (StillenTM), has been reported to possess anti-oxidative and anti-inflammatory activities in experimentally induced

gastrointestinal damage as well as hepatic and pancreatic lesions^[1-4]. DA-9601 is now on the market in South Korea and will be on sale in other Asian countries in the near future. However, despite studies in animals and humans, the detailed cellular mechanism of the pharmacologic actions of DA-9601 is largely unknown.

Chemokines are potential mediators that in many cases can act as a signal for the emigration of blood cells. The C-X-C chemokines are considered the most important mediators for the accumulation of granulocytes^[5,6]. One member of this cytokine family, chemokine interleukin-8 (IL-8), has been shown to be elevated in gastric biopsy samples of patients with *H pylori*-associated gastritis^[5], and is considered to be an important mediator for the initiation of host innate immunity by recruiting granulocytes^[7]. On the other hand, CCL20 is a recently described C-C chemokine (also known as a liver- and activation-regulated chemokine or macrophage inflammatory protein 3 α) that was first identified by screening the GenBank database of expressed sequence tags for novel chemokine molecules^[8]. CCL20 is also expressed in gastric epithelial cells, upregulated by infection with *H pylori*, and implicated in the initiation of host adaptive immunity by regulating recruitment of dendritic cells^[9], effector memory T cells and B cells via CCR6^[10]. Given their potential importance in inflammatory responses, these two chemokines may be good target systems in evaluating the anti-inflammatory efficacy of potential pharmacologic drugs in gastric epithelial cells.

In the current study, we primarily investigated whether TNF- α induces IL-8 and CCL20 genes, as well as their protein products, in human gastric epithelial AGS cells. Although TNF- α is a candidate factor for involvement in inflammation-mediated gastric mucosal injury, the mechanisms of action for this cytokine on gastric epithelial cells are still poorly understood. We next analyzed where DA-9601 acted in the TNF- α -induced inflammatory cascade.

MATERIALS AND METHODS

Reagents and Antibodies

DA-9601 (Lot No. DA-9601-L-07) with 0.42% of active ingredient, eupatilin^[11], was extracted from *A. asiatica* and supplied to this study after HPLC analysis in Dong-A Pharmaceutical Co. Ltd., (Yongin, South Korea)^[2]. Alkaline phosphatase-conjugated rabbit anti-goat IgG, and p-nitrophenyl phosphate tablets, dimethyl sulfoxide, phosphate-buffered saline (PBS), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO). Recombinant human TNF- α goat anti-human IL-8 polyclonal antibody, mouse anti-human CCL20 monoclonal antibody (clone 67310.111), and goat anti-human CCL20 polyclonal antibody were obtained from R&D Systems Inc. (Minneapolis, MN). Rabbit anti-human IL-8 polyclonal antibody was from Endogen Inc. (Woburn, MA). Antibodies against p38 kinase, c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and the antibodies specific to the phosphorylated forms

(pp38, Thr180/Tyr182; pJNK, Thr183 Tyr185; pERK1/2, Thr202/Tyr204) were purchased from Cell Signaling Technology, Inc. (Beverly, MA). SB203580, SP600125, PD98059 and PDTC were purchased from Calbiochem (La Jolla, CA). Anti-human I- κ B α was from Santa Cruz Biotechnology (Santa Cruz, CA).

Report gene construction

IL-8 promoter-luciferase reporter vector (pGL3-pIL-8) was obtained from Dr. J.-S. Chun in Gwangju Institute of Science and Technology (Korea). The CCL20 promoter from -1905 to +30 was amplified from 100 ng of human genomic DNA by PCR under standard conditions with the following primers (restriction sites underlined) pCCL20_forward (*SacI*) 5'-ATACCGAGCTCGGCCAGTCTGGTCTCGAACT-3'; pCCL20_reverse (*HindIII*) 5'-ATACCAAGCTTCTTTAATCAATATTGCAGTT-3' and cloned into the pGL3-basic plasmid (Promega, Mannheim, Germany) to generate pGL3-pCCL20 luciferase vector. pGL3-pCCL20 was sequence verified with an ABI3700 sequencer (ABI, Foster City, CA) before use.

Cell culture

Human gastric epithelial AGS cells and human kidney epithelial 293T were obtained from the American Type Culture Collection (ATCC). The cells were cultured at 37°C in a 5% CO₂ atmosphere in RPMI-1640 supplemented with heat-inactivated 10% fetal bovine serum (FBS; GibcoBRL) and appropriate antibiotics.

Cell viability assay

Cellular viability was evaluated by the reduction of MTT to formazan. A stock solution of MTT was prepared in phosphate-buffered saline (PBS), diluted in RPMI 1640 medium, and added to cell-containing wells at a concentration of 0.5 mg/mL after the culture medium was first removed. The plates were then incubated for 4 h at 37°C in 5% CO₂. At the end of the incubation period, the medium was aspirated, and the formazan product was solubilized with dimethyl sulfoxide. Absorbency was measured on a multiscan reader with a 570 nm wavelength filter.

IL-8 and CCL20 Measurement

The concentration of IL-8 or CCL20 in culture supernatants from AGS cells was measured by a previously described method^[12,13]. In brief, 96-well microtiter plates (MaxiSorp™, Nunc, Denmark) were coated with 2 μ g/mL of goat anti-human IL-8 (R&D Systems) or mouse anti-human CCL20 (clone 67310.111; R&D Systems) in 50 μ L PBS at 4°C overnight. All further steps were carried out at room temperature. After washing three times with PBS, non-specific binding sites were blocked by incubation with 150 μ L PBS, 1% BSA and 0.05% Tween 20/well for 2 h. After three washes with PBS, 50 μ L of samples or standards were added and incubated for 2 h. As a second antibody, 0.5 μ g/mL polyclonal rabbit anti-human IL-8 (Endogen) or polyclonal goat anti-human CCL20 (R&D Systems) was added and incubated for 2 h. As a third antibody, alkaline phosphatase-conjugated monoclonal mouse anti-rabbit IgG (for IL-8) or rabbit anti-goat IgG

(for CCL20) was diluted in 50 μ L PBS 0.1% BSA and 0.05% Tween 20 to 1:50000 and incubated for 2 h. Finally, alkaline phosphatase substrate *p*-nitrophenyl phosphate (Sigma) was added at a concentration of 1mg/mL in 0.1 mol/L glycine buffer, pH 10.4, containing 1 mol/L MgCl₂ and 1 mol/L ZnCl₂. After overnight incubation, plates were read at 405 nm on a microplate reader (Molecular Devices Corp., Sunnyvale, CA). The detection limit of the ELISA was 30 pg/mL.

RNA Isolation and RT-PCR

AGS cells (5×10^6) were grown in 60-mm culture dish and were incubated for 16 h in a fresh medium containing stimuli as indicated. After discarding the growth medium, total RNA was isolated from cells using easy Blue (iNtRON Biotechnology, Daejeon, Korea), following the manufacturer's instructions. Reverse transcription of the RNA was performed using AccuPower RT PreMix (Bioneer, Daejeon, Korea). One microgram of RNA and 20 pmol primers were preincubated at 70°C for 5 min and transferred to a mixture tube. The reaction volume was 20 μ L. cDNA synthesis was performed at 42°C for 60 min, followed by RT inactivation at 94°C for 5 min. Thereafter, the RT-generated DNA (2-5 μ L) was amplified using AccuPower[®] RT PreMix (Bioneer, Korea). The primers used for cDNA amplification were: 5'-ATGACTTCCAA GCTGGCCGTGGCT-3' (sense) and 5'-TCTCAGCCCT CTTCAAAACTTCTC-3' (antisense) for IL-8^[14]; 5'-ATG TGCTGTACCAAGAGTTTG-3' (sense) and 5'-TTACAT GTTCTTGACTTTTTTACTGAGGAG-3' (antisense) for CCL20^[15]; 5'-CGGAGTCAACGGATTTGGTCGTAT-3' (sense), 5'-AGCTTCTCCATGGTGGTGAAGAC-3' (antisense) for GAPDH^[12]. Amplification conditions were denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 30 s for 30 cycles. The expected PCR products were 289 bp (for IL-8), 291 bp (for CCL20), and 306 bp (for GAPDH). PCR products were subjected to electrophoresis on 1.2 % agarose gel and were stained with ethidium bromide.

Assessment of NF- κ B-p65-EGFP nuclear translocation

AGS cells were seeded at 5×10^5 in a 4-well plate 1 d before transfection (50% cell confluency). Cells were transfected with serum- and antibiotics-free RPMI 1640 medium containing 4 μ g/mL Lipofectamine 2000 reagent (Invitrogen) and 1.6 μ g/mL of NF- κ B p65-EGFP vector (provided by Prof. Rainer de Martin, Department Vascular Pharmacology and Thrombosis Research, University of Vienna, Austria). After 5 h of incubation, medium was replaced with RPMI 1640 medium containing 10% FBS and antibiotics. Cells were allowed to recover at 37°C for 20 h and subsequently were stimulated as indicated in the text or figures. Fluorescence images were observed under the Olympus microscopy (Melville, NY).

Cell extract preparation and Western blot analysis

For the analysis of phosphorylated or total protein levels of mitogen-activated protein kinases (MAPKs) and the I- κ B degradation, stimulated cells were rinsed twice with ice-cold phosphate-buffered saline and then lysed in ice-

cold lysis buffer (50 mmol/L Tris-HCl, pH 7.4, containing 150 mmol/L NaCl, 1% Nonidet P-40, 0.1% SDS, 0.1% deoxycholate, 5 mmol/L sodium fluoride, 1 mmol/L sodium orthovanadate, 1 mmol/L 4 nitrophenylphosphate, 10 g/mL of leupeptin, 10 μ g/mL of pepstatin A, and 1 mmol/L 4-(2 aminoethyl) Benzene Sufonyl fluoride). Cell lysates were centrifuged at 15000 rpm for 20 min at 4°C, and the supernatant was mixed with a one-fourth volume of 4 \times SDS sample buffer, boiled for 5 min, and then separated through a 12% SDS-PAGE gel. After electrophoresis, proteins were transferred to a nylon membrane by means of Trans-Blot SD semi-dry transfer cell (Bio-Rad, Hercules, CA). The membrane was blocked in 5% skim milk (1 h), rinsed, and incubated with primary antibody (for phosphorylated MAP kinases or I- κ B) in TBS containing 0.05% Tween 20 (TBS-T) and 3% skim milk overnight at 4°C. Excess primary antibody was then removed by washing the membrane four times in TBS-T, and the membrane was incubated with 0.1 μ g/mL peroxidase-labeled secondary antibody (against rabbit) for 1 h. Following three washes in TBS-T, bands were visualized by ECL[™] Western Blotting Detection reagents and exposed to X-ray film.

Transient transfection and luciferase activity assay

For transient transfections, AGS cell and 293T Cells were seeded at 5×10^5 in a 12-well plate 1 d before transfection (90%-95% cell confluency). Cells were transfected with serum- and antibiotics-free RPMI 1640 medium containing 4 μ g/mL Lipofectamine 2000 reagent (Invitrogen) and 1.6 μ g/mL of NF- κ B, IL-8 (provided by Professor J.-S. Chun, Gwangju Institute of Science and Technology, Gwangju, Korea), or CCL20 luciferase reporter constructs. After 5 h of incubation, medium was replaced with RPMI 1640 medium containing 10% FBS and antibiotics. Cells were allowed to recover at 37°C for 20 h and subsequently were stimulated as indicated in the text or figures. Cell lysates were prepared and assayed for luciferase activity using Luciferase Assay System (Promega, Madison, WI), according to the manufacturer's instructions.

RESULTS

TNF- α induces IL-8 and CCL20 gene expression and protein release in AGS cells

One of the key molecules mediating the gastric mucosal inflammation is the cytokine TNF- α ^[16]. We therefore first examined whether TNF- α induces inflammatory signals in AGS cells. We were primarily interested in two chemokines; i.e., IL-8 and CCL20, because these proteins play central roles in the evocation of host innate and adaptive immunity^[10,17]. Treatment of AGS cells with TNF- α markedly induced IL-8 and CCL20 secretion (Figure 1). The effect of TNF- α was concentration-dependent in the range of 0-10 ng/mL, as assessed by ELISA and RT-PCR. We chose 5 ng/mL of TNF- α for the following experiments as this concentration is enough for maximal induction of IL-8 and CCL20 (Figure 1A and C). Time-dependent experiments revealed that treatment with TNF- α led to rapid induction of IL-8 and CCL20 mRNAs (about 4 h after stimulation) while their

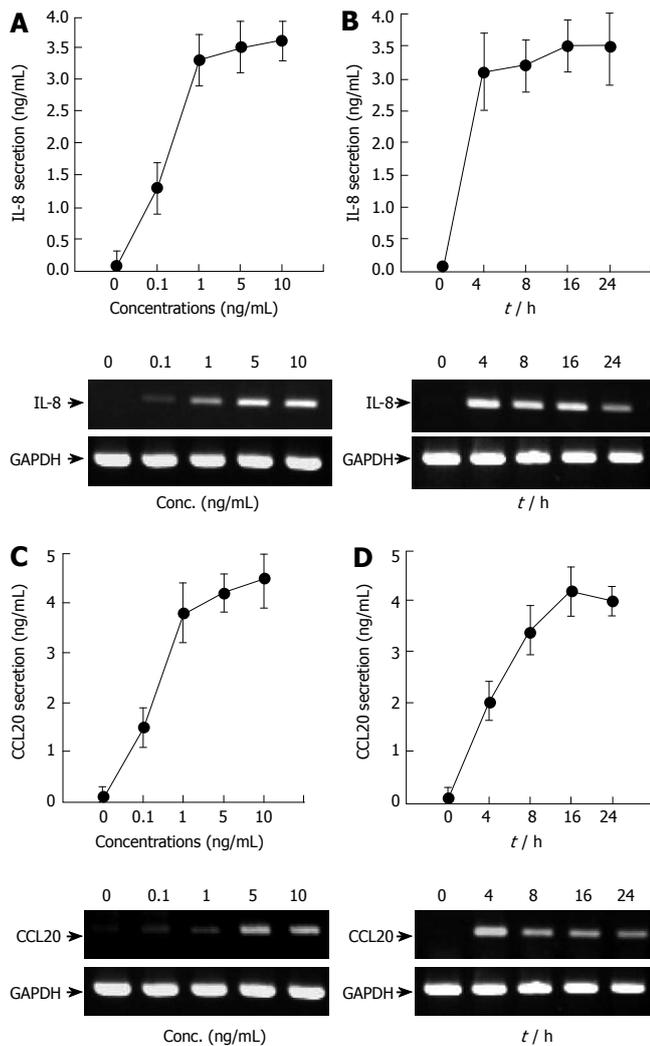


Figure 1 TNF- α induces IL-8 and CCL20 secretion and mRNA accumulation in AGS cells in a time- and dose-dependent manner. (A and C) AGS cells (5×10^5 /well) were treated for 16 h with the indicated concentrations of TNF- α (0-10 ng/mL). After incubation, the supernatants were collected, and the levels of IL-8 (A) and CCL20 (C) were determined by ELISA (top). At the same time, the cells were collected and the expression of two chemokines was determined by RT-PCR (bottom). (B and D) AGS cells (5×10^5 /well) were treated with TNF- α (5 ng/mL) for the indicated time points (0-24 h). IL-8 and CCL20 secretion and mRNA accumulation were determined as described above. For ELISA, results are expressed as means \pm SD of three independent experiments.

protein secretions were slightly delayed (about 16 h after stimulation) in AGS cells. Overall, we conclude that AGS cells produce IL-8 and CCL20 in response to TNF- α .

DA-9601 inhibits TNF- α -induced IL-8 and CCL20 gene expression and protein release in AGS cells

According to previous reports the main effect of DA-9601 is associated with cell death or apoptosis in the rat model of cerulein-induced pancreatitis^[1]. To test preliminarily whether DA-9601 affects viability of human gastric epithelial AGS cells, we performed a MTT assay. The concentrations that ranged from 0 to 100 μ g/mL of DA-9601 showed no toxic effects on AGS cells at 16 h of incubation, while higher concentrations of DA-9601 (>100 μ g/mL) induced delayed cytotoxicity after 24 h (Figure 2A and data not shown). There were no detectable apoptotic nuclei in DA-9601 (0-100 μ g/mL)-treated cells, as verified

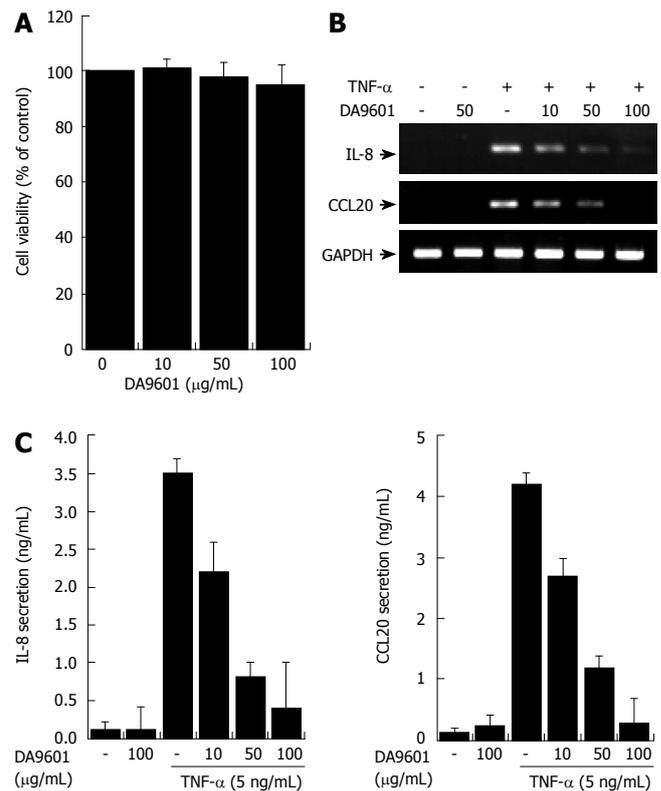


Figure 2 DA-9601 inhibits the expression and secretion of CCL20 in AGS cells. (A) AGS cells (1×10^5) were treated with various concentrations of DA-9601 (0-100 μ g/mL) for 16 h. Quantitative analysis of cell viability was determined by the MTT assay (mean \pm SD, $n = 3$). (B) Cells (5×10^5) were pretreated with various concentrations of DA-9601 (0-100 μ g/mL) for 1 h, and then the cells were further incubated for 8 h with TNF- α (5 ng/mL). Levels of IL-8 and CCL20 mRNAs were determined by RT-PCR. (C) Cells (5×10^5) were pretreated with various concentrations of DA-9601 (0-100 μ g/mL) for 1 h, and then the cells were further incubated for 16 h with TNF- α (5 ng/mL). IL-8 and CCL20 protein levels were determined by ELISA. These data are representative of three independent experiments.

by DAPI staining (data not shown).

RT-PCR revealed that DA-9601 (0-100 μ g/mL), which alone did not induce any significant changes, significantly attenuated TNF- α (5 ng/mL)-dependent expression of IL-8 and CCL20 mRNA in human AGS cells (Figure 2B). Addition of DA-9601 dramatically reduced TNF- α -induced IL-8 and CCL20 secretions as well in a dose-dependent manner (Figure 1C). The concentration of 100 μ g/mL of DA-9601 maximally inhibited the secretion of both chemokines; i.e., IL-8 and CCL20 (Figure 1C). However, as this concentration revealed weak cytotoxicity after 24 h of treatment (data not shown), we therefore chose 50 μ g/mL of DA-9601 for the following experiments, unless otherwise indicated.

DA-9601 inhibits TNF- α -induced IL-8 and CCL20 promoter activities in both HEK293T cells and AGS cells

To investigate whether the inhibition of both chemokine secretions by DA-9601 is due to the direct down-regulation of promoter activity, we performed the luciferase reporter gene assay for IL-8 and CCL20 promoters. As shown in Figure 3, treatment with TNF- α significantly induced IL-8 and CCL20 promoter activities (promoter-dependent luciferase signals) in both HEK293T cells and AGS cells.

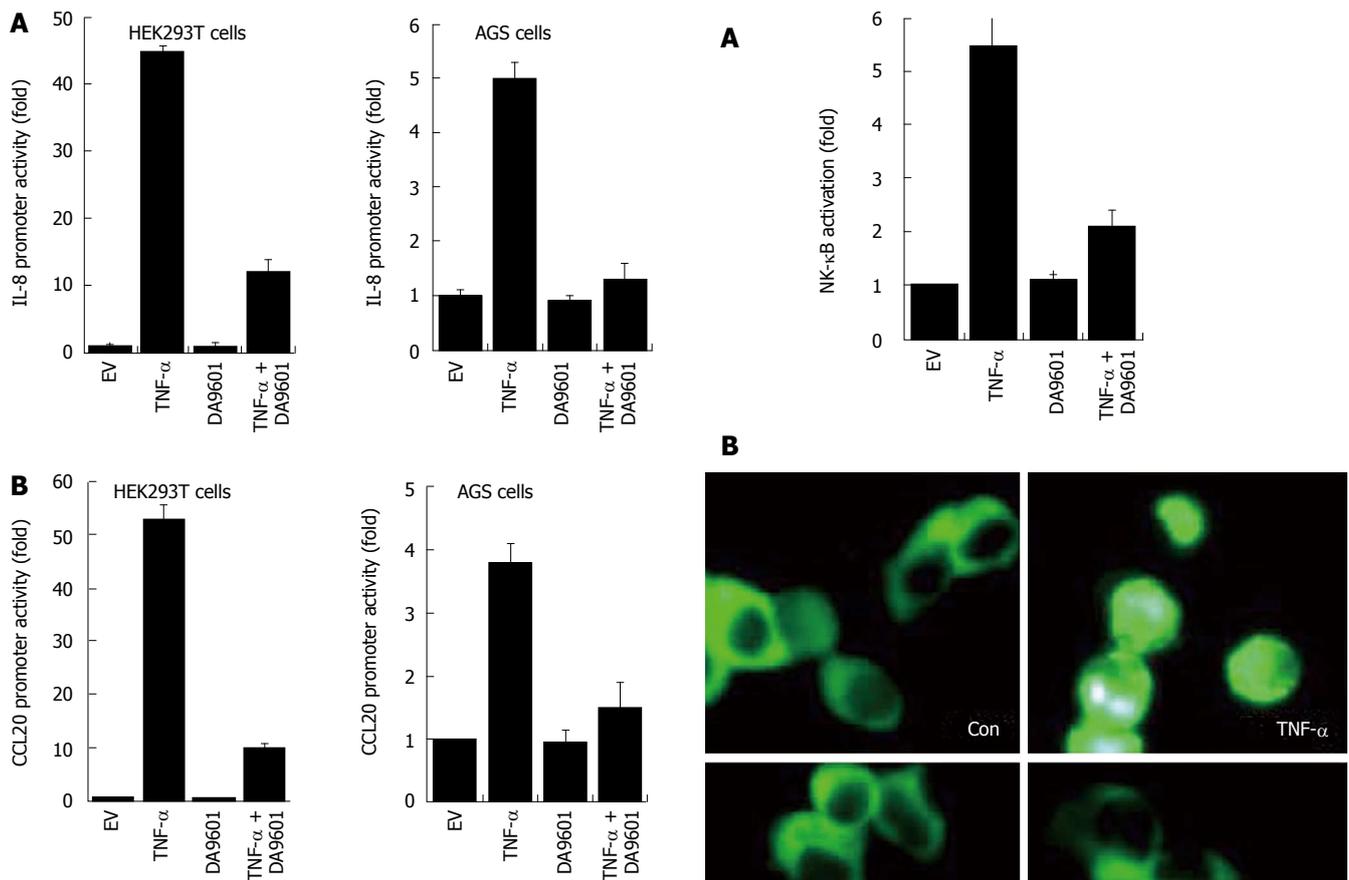


Figure 3 DA-9601 blocks IL-8 and CCL20 promoter activities in HEK293T and AGS cells. HEK293T (left) and AGS (right) cells were transfected with pGL3-pIL-8 (A) or pGL3-pCCL20 (B) luciferase vectors. After 24 h of incubation, cells (1×10^5) were pre-treated for 1 h with DA-9601 (50 μ g/mL) and stimulated for additional 16 h with medium alone or medium containing TNF- α (5 ng/mL). At the end of incubation, cells were lysed, and the relative luciferase activity was measured using Luciferase Assay System. Results are expressed as means \pm SD of three independent experiments.

However, pre-incubation of these cells with DA-9601 (50 μ g/mL) dramatically reduced TNF- α -induced promoter activities in both cell types, suggesting that DA-9601 inhibits IL-8 and CCL20 expressions and secretions *via* direct or indirect modulation of promoter activities.

DA-9601 inhibits TNF- α -induced NF- κ B activity in both HEK293T cells and AGS cells

Several recent studies have demonstrated that gene expression of both IL-8 and CCL20 is NF- κ B dependent^[18-21]. This led us to examine whether DA-9601 can inhibit NF- κ B activity in TNF- α -treated AGS cells. We therefore examined the NF- κ B activation by measuring NF- κ B-dependent transcriptional activity, NF- κ B p65 nuclear translocation, and I- κ B α degradation. As shown in Figure 4A and B, incubation of AGS cells with DA-9601 for 24 h significantly decreased TNF- α -induced luciferase activity. We next measured nuclear translocation of the NF- κ B p65 subunit. To this end, AGS cells were transfected with NF- κ B-p65-EGFP vector for 24 h, and then the cells were further treated with DA-9601 (50 μ g/mL), TNF- α (5 ng/mL), or DA-9601 (1 h before TNF- α treatment) plus TNF- α . As shown in Figure 3C, DA-9601 significantly inhibited nuclear translocation of NF- κ B p65

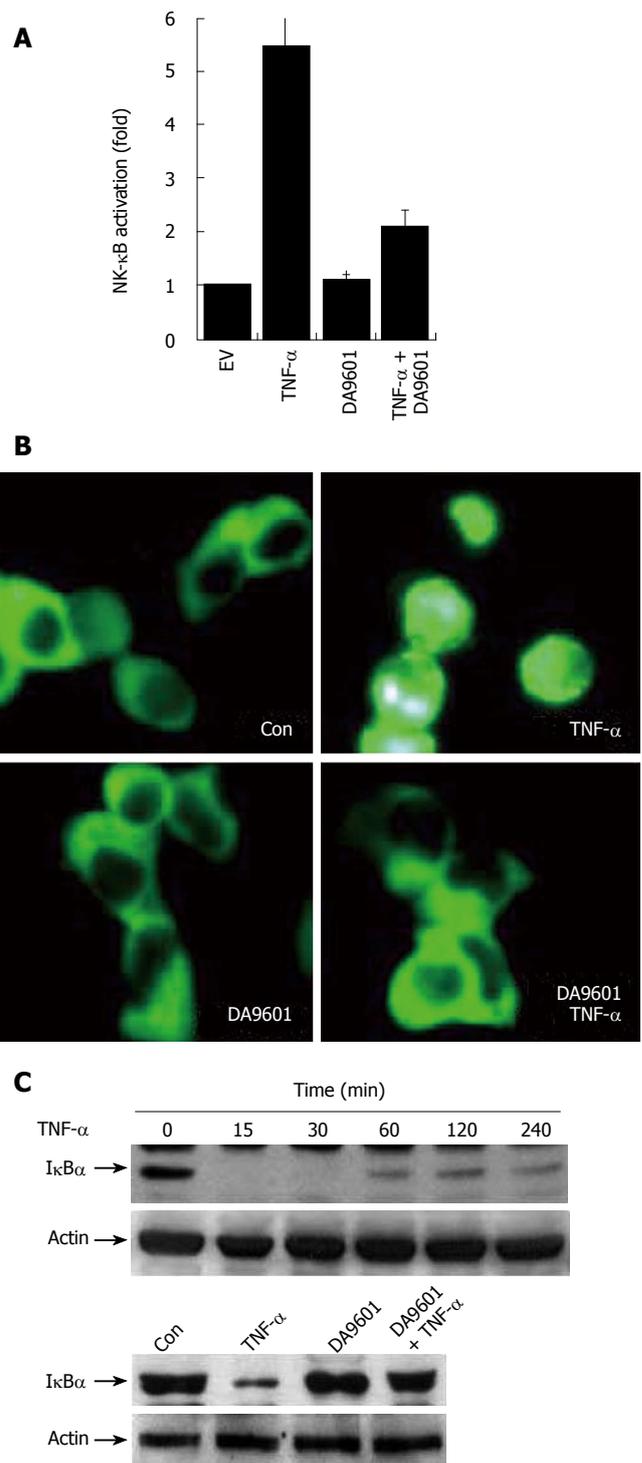


Figure 4 DA-9601 blocks NF- κ B activity in AGS cells. **A:** AGS cells (1×10^5) were transfected with NF- κ B luciferase reporter vector (0.8 μ g/well). After 24 h of incubation, cells (1×10^5) were pre-treated for 1 h with DA-9601 (50 μ g/mL) and stimulated for additional 16 h with medium alone or medium containing TNF- α (5 ng/mL). At the end of incubation, cells were lysed, and the relative luciferase activity was measured using Luciferase Assay System; **B:** AGS cells (1×10^5) were transfected with p65-EGFP vector (0.8 μ g/well). After 24 h of incubation, cells were pre-treated for 1 h with DA-9601 (50 μ g/mL) and stimulated for 1 h with medium alone or TNF- α . Nuclear translocation of p65-EGFP was observed under the fluorescence microscope (original magnification, 200 X); **C:** AGS cells (5×10^5) were incubated with TNF- α (5 ng/mL) for the indicated time points (0-240 min) (top) or were pretreated with medium alone or with DA-9601 (50 μ g/mL) for 1 h, and incubated with TNF- α for 30 min (bottom). The cell lysates were blotted with antibodies specific for the I- κ B α and β -actin.

subunit after 1 h of TNF- α treatment. We finally tested

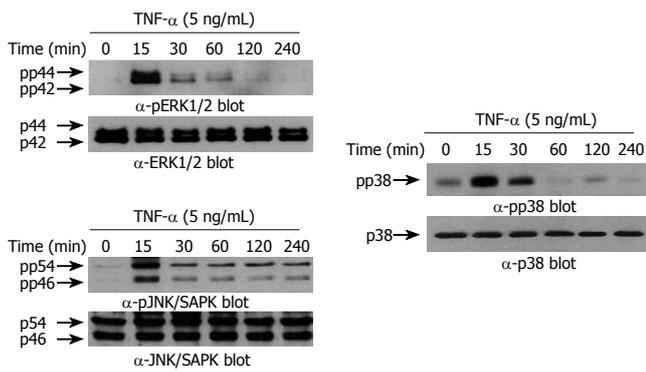


Figure 5 TNF- α induces phosphorylation of MAPKs in AGS cells. AGS cells (5×10^5 cells/well) were incubated for various times (0-240 min) with TNF- α (5 ng/mL). Protein extracts were prepared at the indicated time points, and then the levels of phosphorylated or total MAPKs (ERK-1/2 (top), p38 kinase (middle), and JNK/SAPK (bottom)) were determined by Western blotting using specific antibodies. The arrows indicate the position of specific immunoreactive bands corresponding to distinct MAPKs.

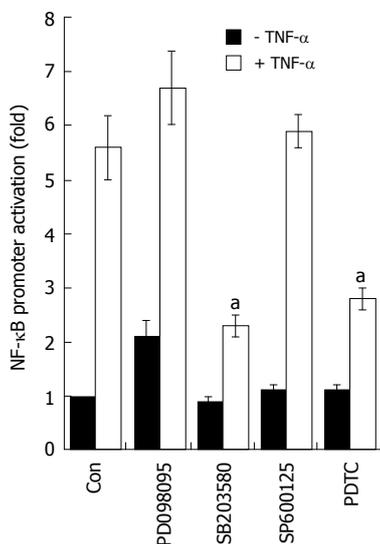


Figure 6 SB203580 blocks NF- κ B-dependent transcriptional activity in AGS cells. AGS cells (5×10^5) were transfected with NF- κ B luciferase reporter vector (0.8 μ g/well). After 24 h of incubation, cells were pre-treated for 1 h with PD098095 (20 μ mol/L), SB203580 (10 μ mol/L), SP600125 (2 μ mol/L), and PDTC (10 μ mol/L); the cells were stimulated for additional 16 h with medium alone or medium containing TNF- α (5 ng/mL). At the end of incubation, cells were lysed, and the relative luciferase activity was measured using Luciferase Assay System. Note, ^a $P < 0.05$, significantly different from control ($n = 4$).

whether DA-9601 inhibits I- κ B α degradation in TNF- α -treated AGS cells. Treatment with TNF- α rapidly induced I- κ B degradation (about 15 min) which later recovered slightly (> 240 min) (Figure 4C). However, pre-incubation of AGS cells with DA-9601 (1 h) significantly inhibited TNF- α -induced I- κ B α degradation (Figure 4B). Taken together, we conclude that DA-9601 inhibits IL-8 and CCL20 expressions and their protein releases, presumably by acting at the site or upstream of NF- κ B-dependent pathways.

p38 kinase plays a crucial role in TNF- α -induced NF- κ B activity as well as CCL20 production in AGS cells

Previous reports demonstrated that three structurally-related mitogen-activated protein kinases (MAPKs) play crucial roles in a variety of systems involving TNF- α ^[22-24]. We therefore asked whether TNF- α leads to phosphorylation of the MAPK subfamilies in AGS cells. As shown in Figure 5, treatment with TNF- α (5 ng/mL) rapidly induced phosphorylation of all three MAPK subfamilies. The maximal phosphorylation levels

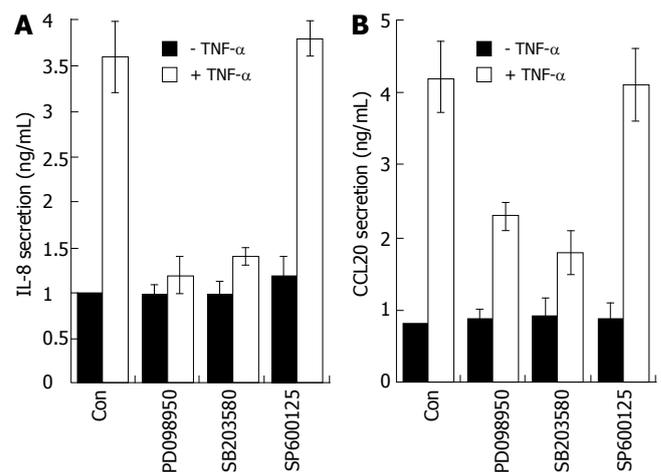


Figure 7 Effects of MAPK modulators on IL-8 and CCL20 release by TNF- α in AGS cells. AGS cells (1×10^5) were pre-treated for 1 h with or without selective MAPK inhibitors (PD098095, 20 μ mol/L; SB203580, 10 μ mol/L; SP600125, 2 μ mol/L). The cells were then further incubated for 16 h with TNF- α (5 ng/mL). Levels of IL-8 and CCL20 protein were determined by ELISA. These data are representative of three independent experiments.

were achieved as early as 15 min in all MAPKs after TNF- α treatment, and thereafter the levels were gradually decreased. Interestingly, however, among three MAPK blockers used, the selective p38 kinase inhibitor SB203580 (10 μ mol/L) could only inhibit TNF- α -induced NF- κ B-dependent promoter activity (Figure 6). As expected, treatment with PDTC (10 μ mol/L) also inhibited NF- κ B-dependent promoter activity (Figure 6). These results suggest a functional cross-talk between p38 kinase and NF- κ B-dependent signaling system, and further suggest that p38 kinase acts upstream of NF- κ B activation, thereby inhibiting IL-8 and CCL20 promoter activities in AGS cells.

To further confirm that p38 kinase is involved in chemokine production, AGS cells were incubated with three MAPK inhibitors prior to TNF- α treatment and then the production of IL-8 and CCL20 was measured by the ELISA method. As expected, treatment with SB203580 significantly inhibited IL-8 and CCL20 production induced by TNF- α (Figure 7). The selective inhibitor of the MEK1 pathway PD098095 also inhibited IL-8 and CCL20 production in TNF- α -treated AGS cells, while it had no effect on NF- κ B-dependent transcriptional activity (Figure 6). These results may suggest that, in terms of IL-8 or CCL20 production, ERK1/2 is not coupled with NF- κ B-dependent pathways but may act at a post-transcriptional level.

DA-9601 inhibits p38 kinase phosphorylation, but shows little effect on ERK and JNK in AGS cells

An important question raised at this point was whether DA-9601 also inhibits p38 kinase phosphorylation induced by TNF- α . To test this, AGS cells were treated with DA-9601 for 1 h, and then the cells were further incubated for 15 min with TNF- α (5 ng/mL). The phosphorylation levels of all three MAPKs were determined by Western blot analysis. Surprisingly, while having no effect on both ERK1/2 and JNK1/2, DA-9601 specifically and

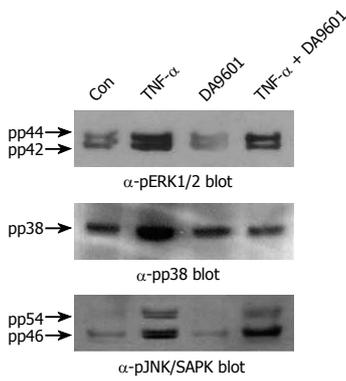


Figure 8 DA-9601 selectively attenuates TNF- α -mediated phosphorylation of p38 kinase in AGS cells. Cells (5×10^5 cells/well) were pretreated for 1 h with medium alone or medium containing DA-9601 (50 μ g/mL). Then, the cells were stimulated for 15 min with or without TNF- α (5 ng/mL). The cell lysates were blotted with antibodies specific for the phosphorylated or total forms of ERK1/2, p38 kinase, and JNK/SAPK, and visualized using a peroxidase-conjugated secondary antibody and the ECL system.

significantly inhibited p38 kinase phosphorylation induced by TNF- α (Figure 8). These results strongly suggest that DA-9601 inhibits TNF- α -induced chemokine production as well as secretion via a direct or indirect inhibition of p38 kinase activity. Further, these results suggest that DA-9601 inhibits NF- κ B-dependent transcriptional activity presumably by blocking the p38 kinase signaling system.

DISCUSSION

In this study we analyzed the effect and mechanism of action whereby DA-9601 modulates the production of two chemokines; i.e., IL-8 and CCL20, in human gastric epithelial AGS cells. IL-8 is one of the key molecules that is involved in innate immunity, and is known to be elevated in gastric biopsy samples of patients with *H pylori*-associated gastritis^[5]. In contrast, it was generally accepted that gastric epithelial cells do not produce the cytokines that are essential components of host adaptive immunity. However, recent study has shown that they do produce CCL20 upon infection with *H pylori*^[9], thereby suggesting that CCL20 is also involved in gastric mucosal immunity. The present results demonstrate that AGS cells do produce both IL-8 and CCL20 chemokines in response to TNF- α stimulation. This evidence extends the current view and suggests that gastric epithelial cells may also have a critical function by inducing mucosal innate immunity as well as adaptive immunity.

The use of natural anti-inflammatory products provides an attractive and safe alternative to modulate inflammatory disorders. *A. asiatica* has been widely used for centuries in traditional Asian diets and medications without any serious side effects. Also, the standardized ethanol extract (DA-9601) of this medicinal plant has been shown to have strong antioxidative and anti-inflammatory effects in experimental animal models, such as esophageal mucosal damage^[23,26], ethanol-induced gastritis^[4], and cerulin-induced pancreatitis^[1]. However, the mechanisms of action of DA-9601 *in vitro* and *in vivo* are still unclear. Our data indicate that TNF- α -mediated expression of chemokine genes in gastric epithelial cells is blocked by DA-9601 treatment. The mechanism of action of DA-9601 involves blockade of NF- κ B activation, in agreement with previous studies using a mouse skin model^[27]. To further define the mechanism by which NF- κ B activity is inhibited by

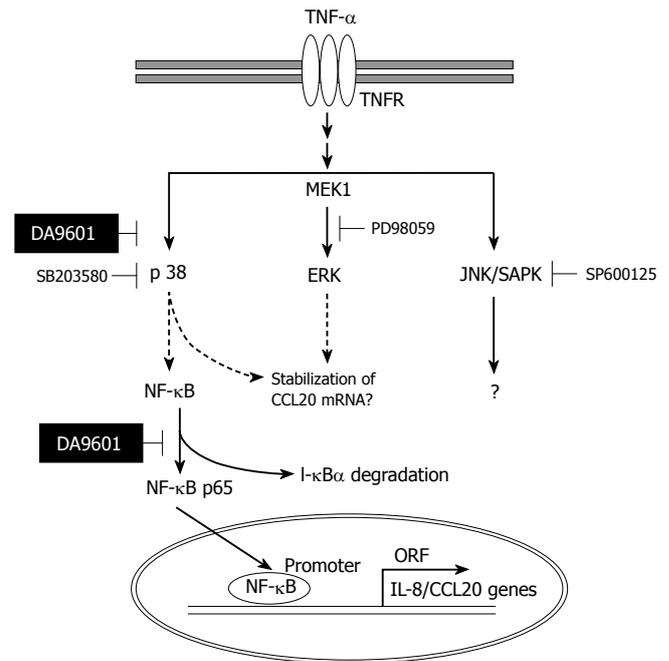


Figure 9 Hypothetical mechanism of action of DA-9601 on TNF- α -induced CCL20 expression in AGS cells. TNF- α induces activation of three MAPKs. Among three MAPKs, however, activation of p38 kinase involves in NF- κ B signaling system. DA-9601 may inhibit NF- κ B directly or indirectly through the inhibition of p38 kinase pathway. See text for discussion.

DA-9601, we investigated molecular relationships between MAPKs and NF- κ B. We found that SB203580, a selective inhibitor of p38 kinase, blocked NF- κ B activity, thereby suggesting that p38 kinase may be functionally linked with NF- κ B. In this regard, it is particularly interesting to note that DA-9601 could block activation of both p38 kinase and NF- κ B in AGS cells. This suggests that DA-9601 does not directly block the NF- κ B-dependent signaling system, but instead, it may indirectly inhibit NF- κ B through the inhibition of p38 kinase pathways. Accordingly, several lines of evidence have suggested that p38 kinase lies upstream of NF- κ B^[28-30]. It is also interesting that PD098059, the upstream inhibitor of ERK1/2 pathway, had no effect on TNF- α -induced NF- κ B activity, while it significantly blocked IL-8 and CCL20 production. These results suggest that the ERK1/2 pathway is not involved in the regulation of promoter activity but may participate in the stabilization of chemokine genes, as demonstrated by other reports^[12,31].

Although DA-9601 has substantial anti-inflammatory or anti-oxidative effects, the structural identity of its active component(s) remains to be elucidated. Eupatilin, one of the major pharmacologically active ingredients of *A. asiatica*, may share its anti-inflammatory^[32] or anti-oxidative effects^[33] with DA-9601. However, our unpublished results demonstrated that eupatilin has no significant effect on TNF- α -induced IL-8 expression and secretion, while it has strong protective (anti-oxidative) effect for AGS cells from hydrogen peroxide-induced cellular damage (data not shown). This implies that DA-9601 may also have other active ingredient(s) that selectively inhibit(s) cytokine-induced expression or release of IL-8 and CCL20 proteins

as well as other inflammation-related proteins.

Collectively, the data obtained in the present study are compatible with the schematic representation in Figure 9. DA-9601 has an anti-inflammatory potential based on its blocking effects on TNF- α -induced IL-8 and CCL20 production. The inhibition by DA-9601 appears to be mediated through the inhibition of promoter activity as well as the NF- κ B-dependent signaling system. Inhibition of p38 kinase by SB203580 blocked NF- κ B activity, suggesting that p38 kinase is functionally linked with NF- κ B and lies upstream of NF- κ B. More interestingly, DA-9601 inhibited activation of both the p38 kinase and NF- κ B-dependent systems. This suggests that DA-9601 inhibits NF- κ B directly or indirectly through the inhibition of the p38 kinase pathway. Additional studies will be required to clarify the upstream signal transduction pathways that might be affected by DA-9601.

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Augmenter of liver regeneration promotes hepatocyte proliferation induced by Kupffer cells

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with the concentration of ALR, suggesting that Kupffer cells play a dual role in liver regeneration.

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Key words: Liver regeneration; Augmenter of liver regeneration; Kupffer cell

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Abstract

AIM: To observe the effects of augmenter of liver regeneration (ALR) on Kupffer cells and to determine whether ALR promotes hepatocyte proliferation induced by Kupffer cells.

METHODS: Kupffer cells and hepatocytes were cultured *in vitro* and various concentrations of recombinant rat ALR (rrALR) were added. ³H-thymidine, BrdU and ³H-leucine incorporation was determined in cultured Kupffer cells and hepatocytes, in hepatocytes conditioned by Kupffer cells, and in associated medium. rrALR was labeled by iodination and used to determine its binding activity by Scatchard analysis in Kupffer cells and primarily cultured rat hepatocytes.

RESULTS: rrALR stimulated DNA replication in Kupffer cells and protein synthesis both in cells and in medium in a non-concentration-dependent manner. The effect was significant at the concentration of 1 µg/L ALR. However, rrALR had no effect on primarily cultured hepatocytes, when hepatocytes were cultured with the Kupffer cell medium conditioned by ALR, DNA replication and protein synthesis in hepatocytes increased significantly at the concentration of 1 µg/L ALR. When the ALR concentration was increased, its effect on hepatocyte proliferation decreased to the basal level. Scatchard analysis indicated the presence of a single class of high affinity receptors with a dissociation constant (*K_d*) of 0.883 nmol/L and a maximum binding capacity (*B_{max}*) of 126.1 pmol/g protein in the rat Kupffer cells.

CONCLUSION: ALR can promote hepatocyte proliferation induced by Kupffer cells, which is associated

INTRODUCTION

Liver regeneration requires various factors promoting cellular proliferation. Among these, augmenter of liver regeneration (ALR)^[1], a novel cytokine, similar to the hepatic stimulator substance^[2], has been shown to be mainly involved in the process of liver regeneration. ALR was cloned by Hagiya *et al*^[1] from the weanling rat liver in 1994. Subsequently, Giorda *et al*^[3] and Yang *et al*^[4] cloned the cDNA of human ALR. It was found that rat, mouse, and human ALR genes (and protein products) are highly conserved, homologous and preferentially expressed in testis and liver^[3]. ALR and the *Saccharomyces cerevisiae* homologue *Erv 1p* are members of the new ALR/*Erv 1* protein family^[5-7]. Experiments and clinical research demonstrated that ALR could specifically promote hepatocyte proliferation and rescue liver failure^[8-13]. The specific molecular impact of ALR on liver regeneration still remains unclear. Wang *et al*^[14] have identified and characterized the receptor for human ALR on rat hepatocytes, and they found that human hepatoma cells *via* their specific receptor ALR may initiate a corresponding cytoplasmic signal transduction. But it was also reported that ALR promotes liver regeneration by inhibiting the activity of hepatic natural killer (NK) cells^[13] and rat ALR (rALR) has no effect on primary rat hepatocytes *in vitro*^[15], suggesting that ALR may likely exert its biological effect by using hepatic non-parenchymal cells. Kupffer cells, the resident liver macrophages, have been implicated as the primary source of inflammatory factors but may also be the source of important growth

factors and cytokines that initiate cellular recovery and regeneration^[16,17]. The protective role of Kupffer cells in hepatic injury by promoting liver regeneration has been recently emphasized^[18-20], but the relationship between ALR and Kupffer cells in liver regeneration has not been investigated. Here we report the existence of ALR receptor in Kupffer cells and show the fact that ALR promotes hepatocyte proliferation induced by Kupffer cells.

MATERIALS AND METHODS

Isolation and primary culture of rat hepatocytes

Hepatocytes were isolated from male Sprague-Dawley rats (weighing 180-250 g, provided by Animal Center of Chinese Academy of Sciences) by *in situ* perfusion of the liver with collagenase (0.5 g/L type I CLSI, Sigma) as described previously^[21]. The viability of the cells was determined by trypan blue exclusion. Preparations with their viability greater than 85% or higher were used. The cells were suspended in Williams' medium E (Sigma) containing 100 mL/L fetal calf serum, 2 mmol/L L-glutamine, 7 mg/L insulin and 10 mmol/L HEPES, plated in 24-well plates (0.125×10^6 cells/well), and incubated in an atmosphere of 95% air and 5% CO₂ at 37°C. The medium was renewed after incubated for 3 h and cells were used for experiments after an overnight incubation.

Preparation of Kupffer cells

Kupffer cells were prepared from the livers of male Sprague-Dawley rats (200-224 g) as described previously^[22]. Briefly, following the collagenase (type IV from 0.25 g/L clostridium histotium, Sigma)/protease (type XIV from 0.5 g/L streptomyces griseus, Sigma) digestion of liver and removal of hepatocytes and cell debris by low speed centrifugation, Kupffer cells and endothelial cells were purified from other non-parenchymal cells by density gradient centrifugation using 300 g/L metrizamide. Finally Kupffer cells were isolated by a centrifugal elutriation procedure^[22]. The viability of Kupffer cell preparation was greater than 95% as determined by trypan blue exclusion. Kupffer cells were suspended in Williams' medium E containing 100 mL/L heat-inactivated fetal calf serum, 5 MU/L penicillin and 5 g/L streptomycin and plated in 24-well culture plates at a density of 1×10^6 cells/well, then plated in 48-well culture plates at a density of 0.5×10^6 cells/well and incubated in an atmosphere of 95% air and 5% CO₂ at 37°C. The medium was renewed after incubated for 3 h and cells were used for experiments after an overnight incubation.

Determination of the effects of Kupffer cells-conditioned medium on hepatocytes

Kupffer cells were washed and placed in serum-free Williams' medium E containing 0.1% bovine serum albumin (BSA, Sigma) and the test ALR concentrations (control, 1, 10, 100 and 1000 µg/L). After 24 h, the medium was filtered (0.2 µm, Gibco) and transferred to the cultured hepatocytes. Hepatocytes were incubated for

24 h with the test agents, the medium was conditioned by Kupffer cells, and various determinations were then made.

³H-thymidine incorporation assay: Determination of DNA replication

Kupffer cells or hepatocytes were rinsed twice with Hanks' buffered saline solution (HBSS), and placed in serum-free Williams' medium E containing 1 g/L BSA and the test ALR concentrations (control, 1, 10, 100 and 1000 µg/L) for 24 h. After the medium was aspirated from Kupffer cells or hepatocytes, a fresh medium containing 37 MBq/L ³H-thymidine (Amersham Pharmacia Biotech, USA) was added to the culture wells. Following an incubation of 4 h at 37°C, the cells were washed with ice-cold HBSS containing 0.1% BSA, treated with 10% ice-cold trichloroacetic acid (TCA) for 10 min, and washed once with TCA followed by 95% ethanol. The cells were then digested with 5% sodium dodecyl sulfate (SDS) at 60°C for 25 min. The liquid was transferred into scintillation vial, and washed once more with 0.5 mL 0.5% SDS. The radioactivity was determined using 5 mL β-scintillation solution.

³H-leucine incorporation assay: Determination of protein synthesis

Kupffer cells or hepatocytes were stimulated for 24 h as in the ³H-thymidine incorporation assay. After the medium was aspirated, a fresh medium containing 37 MBq/L ³H-leucine (Amersham Pharmacia Biotech, USA) was added to the culture wells. Following an incubation of 4 h at 37°C, the medium aspirated from Kupffer cells or hepatocytes was transferred to 1.5 mL Eppendorf tubes, BSA (finally 5%) and TCA (finally 5%) were added for 15 min, centrifuged at 12900 r/min for 20 min at 4°C, washed three times with 5% ice-cold TCA. Then 200 µL 0.1 mol/L NaOH was added to dissolve the pellet, pH was adjusted with 0.1 mol/L HCl, the liquid was transferred to the scintillation vial. The cells were washed with ice-cold HBSS containing 1 g/L BSA, treated with 100 g/L ice-cold TCA for 10 min, and washed once with TCA followed by 95% ethanol. The cells were then digested with 5% SDS at 60°C for 25 min. The liquid was transferred to scintillation vial, and washed once with 0.5 mL 0.5% SDS, the radioactivity was determined using 5 mL β-scintillation solution.

BrdU incorporation assay

Cells were plated in 12-well plates, serum-starved, and stimulated as described previously in the ³H-thymidine incorporation assay. After 24 h of stimulation, the cultures were incubated with BrdU labeling reagent (Dako) diluted at 1:1000. Cells were rinsed and fixed for 30 min at room temperature with acetic alcohol (90% ethanol, 50 mL/L acetic acid, 50 mL/L distilled H₂O). Endogenous peroxidase was blocked by incubation in 1% H₂O₂ in methanol for 20 min at room temperature. Cells were washed in PBS and incubated for 15 min in 1.5 mol/L HCl at 37°C, and extensively washed in PBS. Cells were then incubated in anti-BrdU antibody (mouse IgG, Sigma) diluted at 1:40 in PBS containing 1% BSA for 1 h at 37°C and washed in PBS. Cells were incubated in anti-mouse secondary antibody (goat anti-mouse IgG, Sigma) diluted

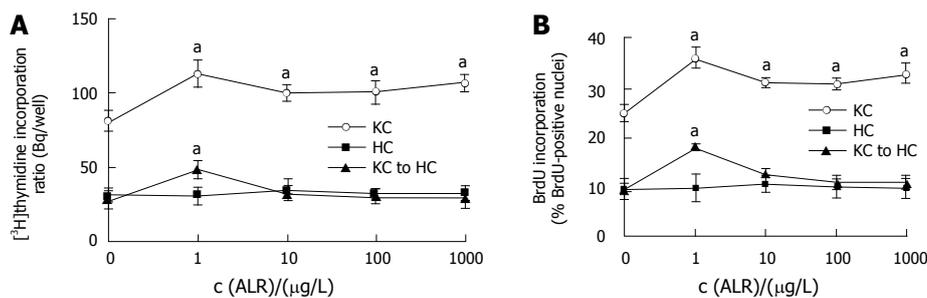


Figure 1 Effects of ALR on proliferation of Kupffer cells and hepatocytes. **A:** DNA replication; **B:** BrdU-positive nuclei. KC: Kupffer cells; HC: Hepatocytes; KC to HC: Hepatocytes conditioned by Kupffer cells. $^*P < 0.05$ vs control, $n = 3$.

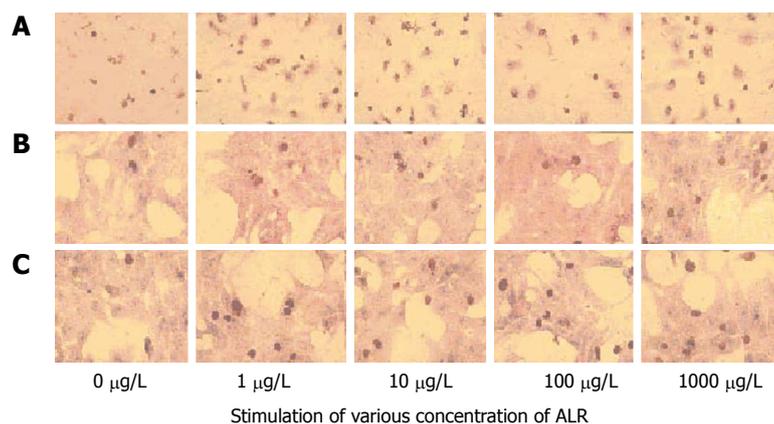


Figure 2 BrdU immunohistochemistry. The nuclei of BrdU-positive cells were stained brown. **A:** Kupffer cells; **B:** Hepatocytes; **C:** Hepatocytes conditioned by Kupffer cells ($\times 100$).

at 1:100 in PBS, and 10 g/L BSA for 30 min at room temperature followed by washing. Cells were incubated in ABC solutions for 30 min at room temperature, washed and incubated in 50 mmol/L Tris (pH 7.6) for 5 min, then the 3, 3'-diaminobenzidine solution was added. Three areas in each well were counted for a total of about 1000 cells. Proliferation was indicated as a percentage of labeled nuclei.

¹²⁵I- recombinant rat ALR binding to cultured kupffer cells and primary hepatocytes

Recombinant rat ALR (rrALR) was expressed in *Escherichia coli* and prepared with high purity (> 95%) as described previously^[2,3]. rrALR was radioiodinated with the chloramines-T methods^[14,24]. Briefly, 15 µL of 50 mmol/L sodium phosphate buffers (pH7.0) and 18.5 MBq/L sodium ¹²⁵I (Amersham Pharmacia Biotech, USA) were added to a siliconized tube containing 5 µg of rrALR. The reaction was started by adding 10 µL of chloramine-T reaction using 20 µL of ending solution (50 nmol/L N-acetyl-L-tyrosine, 0.01 mol/L sodium phosphate buffer). ¹²⁵I-rrALR was separated from free iodine by gel filtration on a column (20 cm \times 11.0 cm) of Sephadex G-25 (Amersham Pharmacia Biotech, USA) equilibrated with PBS and 1 g/L BSA. The fractions containing ¹²⁵I-rrALR were pooled. The assay was performed as described previously^[14, 23]. Kupffer cells and rat hepatocytes were washed in HBSS (containing 20 mmol/L HEPES, 1.3 mmol/L CaCl₂, 0.5 mmol/L MgCl₂, 1% BSA, pH 7.0), and pre-incubated in the presence of the same buffer for 30 min at 25°C. After equilibration was achieved in this medium, 0.3 mL containing 12.5 pmol/L-3200 pmol/L ¹²⁵I-rrALR with or without 5 µmol/L unlabeled rrALR (saturation binding) was incubated for 3 h at 25°C with

constant shaking. The cells were washed 4 times with ice-cold PBS and digested with 0.5 mL 0.75 mol/L NaOH for 30 min at 37°C, and the radioactivity in the digest was determined in a gamma-counter (cpm). Specific binding of ¹²⁵I-rrALR was calculated as the difference between cell-associated radioactivity in the presence and absence of unlabeled rrALR.

Statistical analysis

The values are presented as mean \pm SD of triplicate determinations. Each experiment was repeated at least three times. Student's *t*-test was employed for statistical comparison of the paired samples. $P < 0.05$ was considered statistically significant.

RESULTS

Effects of ALR concentration on Kupffer cells

³H-thymidine incorporation assay showed that rrALR stimulated DNA replication in Kupffer cells in a non-concentration-dependent manner (Figure 1A). The significant effect was observed at the concentration of 1 µg/L ALR, the DNA replication increased to 144.2% as compared to the control (117 ± 11.7 Bq/well *vs* 81.2 ± 6.7 Bq/well, $P < 0.05$). Following the increase of added ALR concentration, DNA replication began to decrease, but was still higher than that in the control ($P < 0.05$). Being consistent with the ³H-thymidine incorporation assay, cell labeling by BrdU increased to 36% from a basal level of 24% at the concentration of 1 µg/L ALR ($P < 0.05$, Figure 1B, Figure2). Similar to the DNA replication, ALR also produced a non-concentration-dependent transforming of protein synthesis both in Kupffer cells and in associated medium, but the significant effect was observed at the

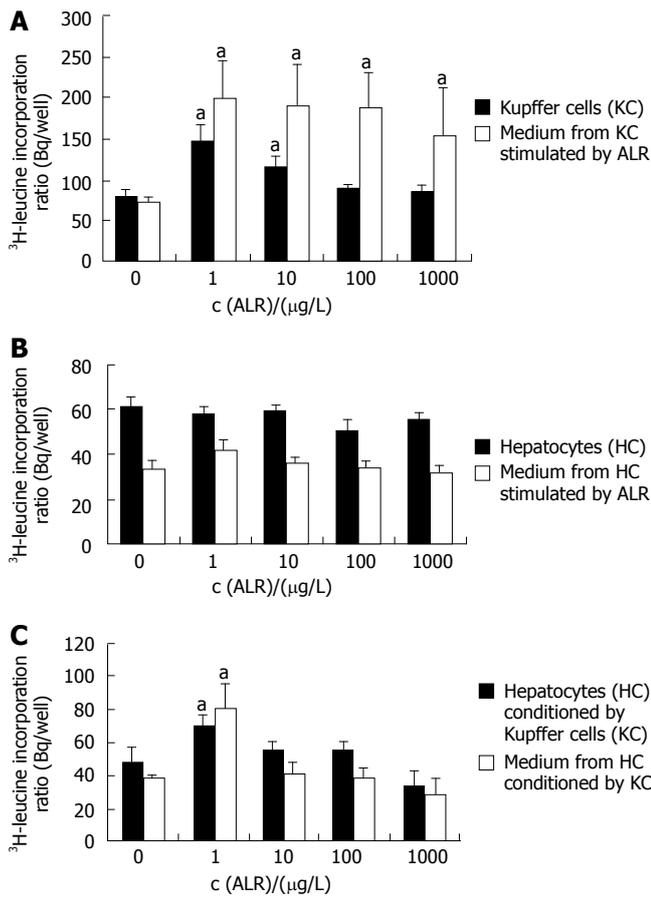


Figure 3 ³H-leucine incorporation assay. **A:** Kupffer cells and associated medium; **B:** Hepatocytes and associated medium; **C:** Hepatocytes conditioned by Kupffer cells and associated medium. **P* < 0.05 vs control, *n* = 3.

lowest concentration of 1 μg/L ALR tested (Figure 3A). The effects in cells and associated medium elevated to 182.6% and 275.3%, respectively, as compared to those in the control (cells: 147.5 ± 19.6 Bq/well vs 80.8 ± 5.9 Bq/well, *P* < 0.05; associated medium: 198.0 ± 46.1 Bq/well vs 71.9 ± 7.5 Bq/well, *P* < 0.05). Following the increase of added ALR concentration, protein synthesis in associated medium decreased, but was still higher than that in the control (*P* < 0.05, Figure 3A).

Effects of ALR concentration on hepatocytes

ALR did not cause any significant change in DNA replication and protein synthesis in hepatocytes or in associated medium (Figures 1A and B, Figure 2, Figure 3B).

Effects of the medium conditioned by Kupffer cells with ALR on hepatocyte proliferation

Stimulation of hepatocytes with the medium conditioned by Kupffer cells with ALR increased DNA replication and protein synthesis at the lowest concentration of 1 μg/L ALR tested, to 174.3% (³H-thymidine incorporation, Figure 1A) or 180% (BrdU labeling, Figure 1B, Figure 2), 146.2% (in cells) and 210.7% (in associated medium) (Figure 3C) respectively, as compared to that in the control (DNA replication: 48.4 ± 3.5 Bq/well vs 27.8 ± 4.7 Bq/well in ³H-thymidine incorporation, 18.0% vs 10.0% in BrdU labeling; protein synthesis: 70.5 ± 5.7 Bq/well vs 48.2 ± 9.2 Bq/well in cells, 80.7 ± 15.0 Bq/well vs 38.3 ± 2.5 Bq/well in associated medium, *P* < 0.05). But with the increase of added ALR concentration, the medium conditioned by Kupffer cells with ALR produced a concentration-dependent inhibition of DNA or protein synthesis in hepatocytes, the significant effect was observed at the highest concentration of 1000 μg/L ALR tested (DNA: 28.9 ± 5.8 Bq/well, Figure 1A; protein synthesis: 33.9 ± 9.0 Bq/well in cells, 29.3 ± 8.9 Bq/well in the medium, Figure 3C).

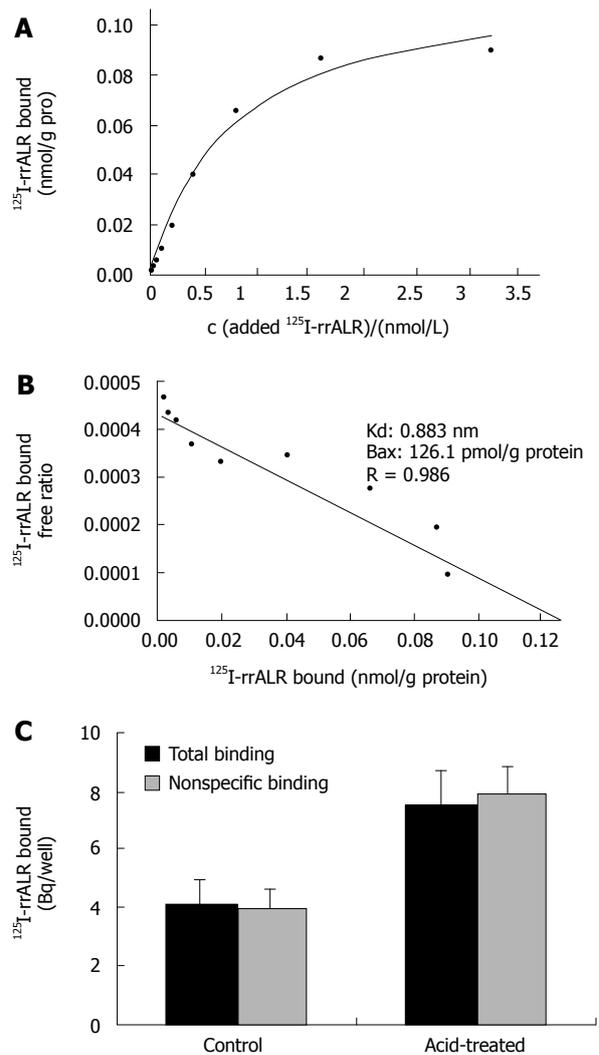


Figure 4 ¹²⁵I-rrALR binding assay. **A:** Saturation curves of ¹²⁵I-rrALR binding to its receptor on Kupffer cells; **B:** Scatchard plot of the ALR receptor on Kupffer cells; **C:** Binding of ALR to cultured hepatocytes (mean ± SD, *n* = 3).

¹²⁵I-rrALR binding assay

Typical saturation curves of ¹²⁵I-rrALR binding to cultured Kupffer cells are shown in Figure 4A. Specific binding of ALR was saturated at about 1.5 nmol/L. Scatchard analysis resulted in a rectilinear plot (Figure 4B), thereby suggesting the presence of a single class of high affinity binding sites, namely the existence of a receptor of ALR on Kupffer cells. The ¹²⁵I-rrALR binding capacity to cultured Kupffer cells was 126.1 ± 22.3 pmol/g protein, the affinity of the receptor was 0.883 ± 0.056 nmol/L.

However, similar to Gandhi *et al*^[23], no receptor for

ALR was found on hepatocytes by radioligand binding analysis. To investigate the possibility that ALR receptors on hepatocytes are down-regulated, the acidic medium, a procedure known to dissociate bound peptide ligand from its receptors, was used to treat cultured hepatocytes as previously described^[21,22], but it did not unmask any specific binding of ALR yet (Figure 4C).

DISCUSSION

Previous studies have revealed that ALR appears to be an important regulator of liver regeneration. Recently, emphasis has been placed on ALR molecular mechanisms underlying liver regeneration. Many advances have been gained in murine and human experiments. For instance, Yang *et al*^[4] have cloned the cDNA of human ALR (hALR) from human fetal liver lysates encoding 125 amino acids and 15KD in molecular weight. hALR could stimulate proliferation of cultured hepatocytes as well as hepatoma cells *in vitro* and rescue acute hepatic failure *in vivo*. ALR exerts its hepatrophic activities through paracrine and autocrine pathways. Wang *et al*^[14] have demonstrated the existence of hALR specific receptor on the surface of primary hepatocytes and hepatoma cells. Through the ALR receptor, ALR stimulates hepatocyte proliferation by activating the mitogen-activated protein kinase cascade (MAPK signaling pathway)^[25]. The existence of ALR in nuclei and cytosol of liver tissues further implicates that ALR has intracellular function in hepatocytes^[26]. In immunohistochemical studies, Thasler *et al*^[27] found that hALR is mainly expressed both in normal and in impaired hepatocytes. Even though there exist many inconsistent issues, Gandhi *et al*^[23] have failed to show any specific rALR receptor in hepatocytes. Hagiya *et al*^[11] and Yu *et al*^[12] reported that rat ALR has no effect on primary rat hepatocytes *in vitro* and that the expression of ALR is absent in normal hepatic tissues^[15]. The existence of contrary results together with the mechanism of inhibiting hepatic NK activity to promote liver regeneration suggests that it is possible for ALR to exert its bioactivities by interacting with other cytokines or with hepatic non-parenchymal cells on hepatocytes.

In this study, we showed the presence of high affinity receptors of ALR on hepatic Kupffer cells, and found that ALR could stimulate proliferation of Kupffer cells, suggesting that ALR stimulates hepatocyte proliferation by activating Kupffer cells, which may improve our understanding of the molecular mechanism of the biological action of ALR and its effect on liver regeneration. ³H-thymidine and ³H-leucine incorporation assay and BrdU labeling demonstrated that ALR stimulated DNA and protein synthesis of Kupffer cells in a non-concentration-dependent manner and had no effect on primary hepatocytes. But with the medium conditioned by Kupffer cells, ALR promoted hepatocyte proliferation. Similar to this study, Chen *et al*^[28] reported that when hepatocytes are co-cultured with Kupffer cell supernatant only activated by LPS, hepatocyte DNA and protein synthesis are increased significantly. Recently Armburst *et al*^[19] reported that the early gene expression

of hepatocyte growth factor in Kupffer cells after carbon tetrachloride (CCl₄) treatment may be an important event in the early phase of liver regeneration. Together with the finding about the entry of ALR into Kupffer cells by immunolabelling^[23], these events suggest that the medium containing some kind of hepatocyte growth factors coming from Kupffer cells conditioned by ALR and secreting into the medium, can regulate hepatocyte proliferation. The increased ³H-leucine incorporation showed the protein synthesis both in Kupffer cells and in associated medium. What kind of protein is needed should be further studied. The foregoing studies have tested acute reactant cytokines such as interleukin-6 (IL-6) and tumor necrosis factor (TNF)- α originated mainly from activated Kupffer cells which are the chief growth factors in hepatocyte proliferation *in vivo*^[17,29-33]. Since TNF- α and IL-6 are involved in the initiation of liver regeneration, we assume that ALR, like LPS, may activate Kupffer cells by unknown means and then mediate Kupffer cell-dependent release of TNF- α and IL-6, triggering hepatocyte proliferation. The ALR receptor on Kupffer cells can elucidate the effect of ALR on Kupffer cells in part. Here, the high affinity receptor for ALR on Kupffer cells can be identified by ¹²⁵I-ALR binding assay. When ALR binds to the cell surface receptor, Kupffer cells are activated and then release various cytokines initiating liver regeneration. The binding effect is saturable. When an excess amount of unlabeled ALR is added, it could replace the cell surface sites to which ALR has bound. It was reported that ¹²⁵I-ALR is found to have the similar molecular weight and biological activity as the unlabeled ALR, and that iodination does not change the characteristics of natural ALR, suggesting that ¹²⁵I-ALR can be used for identification of ALR receptor^[14]. As for the specificity of this binding, further researches need to be done. In our study, as the concentration of ALR was added to 1000 μ g/L, the effect of ALR-dependent-Kupffer cells on hepatocyte proliferation was inhibited, suggesting that Kupffer cells have a dual function as a regulator in liver regeneration. Responding to the immoderate proliferation effect of hepatocytes, Kupffer cells exert their inhibitory effects by releasing growth inhibiting factors. Transforming growth factor(TGF)- β is the most obvious candidate because it has been shown *in vivo* models that TGF- β could antagonize TNF- α actions and induce apoptosis, constraining the amount of hepatocyte proliferation^[34-36].

Similar to the study by Gandhi *et al*^[23], no receptor for rALR was found on hepatocytes in our study, which may interpret the inability of rALR to exert a mitogenic effect on hepatocytes *in vitro*. To investigate the possibility that ALR receptors on hepatocytes are down-regulated, the acidic medium, a procedure known to dissociate bound peptide ligand from its receptors was used to treat cultured hepatocytes, but it did not unmask any specific binding of ALR. At the same time, there is evidence that hepatocytes synthesize and secrete ALR^[23,37]. A further finding showed that hepatic ALR levels decreased while circulating ALR levels increased 12 h after 70% hepatectomy, suggesting that the release of stored ALR from hepatocytes rather than the accelerated synthesis increases the circulating

ALR level^[28].

How should these events be put together? We hypothesize that under pathological or biological circumstances of liver regeneration such as partial hepatectomy or weaning livers, activated Kupffer cells release various cytokines initiating liver regeneration, and that the receptor of ALR on hepatocytes (assuming its existence) and ALR which are constitutively expressed and stored in hepatocytes in an inactive form beforehand, are released from the cells in an active form by unknown means, then ALR exerts its hepatrophic activities through paracrine and autocrine pathways such as binding to Kupffer cells or the inhibition of hepatic NK cells to promote hepatocyte proliferation. To prevent excess hepatocyte proliferation, anti-hepatotrophic growth factors such as TGF- β are produced.

In conclusion, the mechanisms of liver regeneration are involved in complicated interactions of various cytokines and cells^[29,37]. Kupffer cells contain high affinity receptors for ALR on cell surfaces, by which ALR promotes hepatocyte proliferation. Further studies focusing on the examination of the specificity of receptors on Kupffer cells are needed.

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BASIC RESEARCH

Differentiation of hepatocytoid cell induced from whole-bone-marrow method isolated rat myeloid mesenchymal stem cells

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Abstract

AIM: To explore the expansion and differentiation of hepatocytoid cell induced from myeloid mesenchymal stem cell (MSC) *in vitro*, in order to find suitable resource of hepatocytes for bioartificial liver or liver transplantation.

METHODS: The rat myeloid MSC was isolated and divided into three groups which were cultured by Friedenstein method, and then were induced by culture fluid, culture fluid plus cholestatic serum and culture fluid plus hepatocyte growth factor (HGF), respectively. Hepatocytoid cell as well as expression of CK18 and AFP was observed by immunohistochemistry.

RESULTS: After the induction for 21 d, hepatocytoid cell was observed, and its expression of CK18 and AFP was detected by immunohistochemistry in MSC cultured with cholestatic serum. Furthermore, on the 35th d, albumin mRNA was expressed in the cell, suggesting the inducing effect was similar to that by HGF.

CONCLUSION: Rat myeloid MSC can differentiate into hepatocyte lineage under appropriate condition. This method is easy to operate.

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Key words: Mesenchyme stem cell; Hepatocytoid cell; Expansion; Differentiation; Induction

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INTRODUCTION

Over the past few years, great progress has been made in the research of stem cell. It has been proved that some stem cells in bone marrow can differentiate into various kinds of cells. Therefore, through culturing myeloid stem cells *in vitro*, and providing suitable conditions, these cells can be induced to generate and differentiate into other cells. There have been some reports that application of cytokines such as hepatocyte growth factor (HGF), fibroblast growth factor-4 (FGF-4) might promote myeloid stem cells to proliferate and differentiate into hepatocytoid cell. However, the cytokines are costly. This study was to explore a convenient way to induce myeloid mesenchyme stem cell (MSC) into hepatocytoid cell *in vitro*.

MATERIALS AND METHODS

Materials

One-month-old Wista rats were provided by the Animal Center of Tongji Medical College, Huazhong University of Science and Technology. Rat recombinant hepatocyte growth factor (HGF) (R&D Co.), Dulbecco's minimum essential medium (DMEM) (Gibco), fetal bovine serum (FBS, Hyclone), N-2-hydroxyethylpiperazine-N'-2'-ethanesulfonic acid (HEPES), galactose solution of insulin, transferrin and selenite (ITS) (Sigma), nicotinamide, trypsinase (Gibco), proline (Wuhan Lingfei Bio. Co.), alpha fetoprotein (AFP) monoclonal antibody (MoAb) (Santa Cruz Co.), cytokinin 18 (CK18) MoAb, biotin labeled Ab, diaminobenzidine (DAB), streptavidin peroxidase (S-P) (Beijing Zhongshan Co.), deoxy-ribonucleoside triphosphate (dNTPs), Pyrotest Taq enzyme (Bao Bio Ltd. Co.), Bio-11-dUTP (Huamei Biotech Co), Deion formamide, salmon sperm DNA (Sigma), fluorescein isothiocyanate (FITC) labeled antibiotin (Boshide) and albumin primer 5'ATACACCCAGAAAGCACCTC3', 5' CACGAATTGTGCGAAGTC AC3' (Shengong Co) were prepared.

Methods

Isolation and culture of myeloid MSC: According to the literature^[1], rat femurs were separated under aseptic condition. The myeloid cell, after doused and blew out from the bone with culture fluid (5 mL DMEM + 10% FBS), was added to the culture fluid to make cell suspension. Then the culturing was begun with the supplementation of fluid of DMEM, 10% FBS, 100 U/

mL penicillin and 100 U/mL streptomycin, at 37°C, in 50 mL/L CO₂ environment. On the third day non-adherent cell was removed and the remaining cells were allowed to grow. On the 10th d the confluent cells were trypsinized and passaged. When the laminating cell with typical cellular appearance was observed, 0.25% trypase was added for digestion for 5-10 min and then the whole medium was added to terminate the digestion. When the inoculation density was $1 \times 10^4/\text{cm}^2$, the nutrient medium was added and the serial subcultivation was begun. The above procedure was repeated to passage for every 3-4 d.

MSC differentiation potency detection: The third generation MSC was inoculated onto 6-well culture plate (density: $5 \times 10^4/\text{mL}$), and the induction culture fluid (0.05 g/L vitamin C, 40 ng/mL dexamethasone, 10 mmol/L β sodium glycerophosphate) was added. Then the cell was passaged for every 4 d. On the 15th d autoclaved coverslips were put into the media and 3 d later the coverslips were taken out and were stained by alkaline phosphatase.

Cholestatic serum preparation: Male rats with weight of 400 g were anesthetized with Pentothal and their abdomens were degermed. After the abdominal cavities were opened, their choledochuses were ligated and then the bellies were sutured. Given normal feed for 10 d, those rats were dissected again at abdomen to obtain blood from inferior caval vein. The serum was separated and preserved in refrigerator at -40°C.

Group division: Three groups, including MSC culture group (only with culture fluid), MSC cultured with cholestatic serum (5%) group and MSC cultured with HGF (0.5 mg/mL) group, were divided. The culture fluid composed of DMEM, 10% FBS, 15 mmol/L HEPES, 10 mmol/L niacinamide, 1 mmol/L vitamin C, 10^{-7} mol/L dexamethasone, 1 mg/mL galactose, 30 $\mu\text{g}/\text{mL}$ proline, ITS and antibiotics. All the cells were inoculated onto 6 well culture plate and allowed to creep on the plate coverslips custodited with polylysine (0.025%). The morphology of the cell was observed on the 7th, 14th, 21st and 35th d according to the literature^[1] and during those days the coverslips were taken out and fixed with paraform (4%) for 30 min.

Detection methods: The expression of CK18 and AFP by the cells was detected by immunohistochemistry and S-P method, with PBS instead of 1st-antibody in negative control. The albumin expression was detected by *in situ* hybridization. Biotin random primer labeling method was applied as albumin labeling probe.

RESULTS

Observation of cell growth condition

Growth condition of MSC culture group: The primary cells adhered to the wall after cultured for 48 h, and the adhered cells increased gradually afterward. After 3 passages, on the 14th d, the cells showed uniform morphology (Figure 1) like fibroblast and grew as grass bunch or whirlpool.

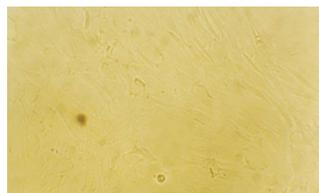


Figure 1 After 3 passages, on the 14th d, the cell showed uniform morphology: fusiform shape or polygon shape, like fibroblast and grew as grass bunch or whirlpool (100 \times).

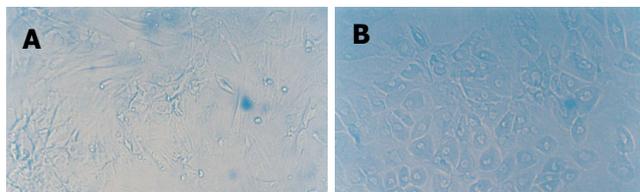


Figure 2 Growth condition of MSC cultured with cholestatic serum. **A:** Cultured with cholestatic serum for 7 d, peripheral cell of the clone showed cuboidal morphology, which is characteristic of hepatocytes (100 \times); **B:** Cultured with cholestatic serum for 35 d, all MSCs showed cuboidal morphology, which is characteristic of hepatocytes (100 \times).

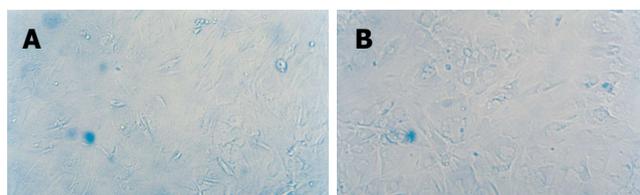


Figure 3 Growth condition of MSC cultured with HGF. **A:** Cultured with HGF for 7 d, peripheral cell of the clone showed cuboidal morphology, which is characteristic of hepatocytes(100 \times); **B:** Cultured with HGF for 35 d, all MSCs showed cuboidal morphology, which is characteristic of hepatocytes (100 \times).

Growth condition of MSC cultured with cholestatic serum group:

From the 35th d, the cell shape changed from fusiform to polygon and the proportion of the polygon cell increased with time. Meanwhile, the cell proliferation was also slowed. These phenomena appeared first in the periphery of the cell clone, and gradually extended into the whole cell clone (Figure 2).

Growth condition of MSC cultured with HGF group:

The characteristics of the cell in this group were similar to that in MSC cultured with cholestatic serum group (Figure 3).

MSC identification

After induced by vitamin C, dexamethasone and β sodium glycerophosphate, respectively, the cells gradually changed to different morphological forms, like triangle, fusiform and polygon. With the prolongation of culturing time, the cell population increased and cell volume enlarged. On the 15th d, the cell endochylema was full and the cells connected with each other with ecphyma. Alkaline phosphatase staining showed positive (Figure 4). This verified that the cells isolated were MSCs.

Expression of CK18 and AFP

Expression of AFP by the cell was observed on the 14th d and expression of CK18 was detected on the 21st d in MSC cultured with cholestatic serum. And these two

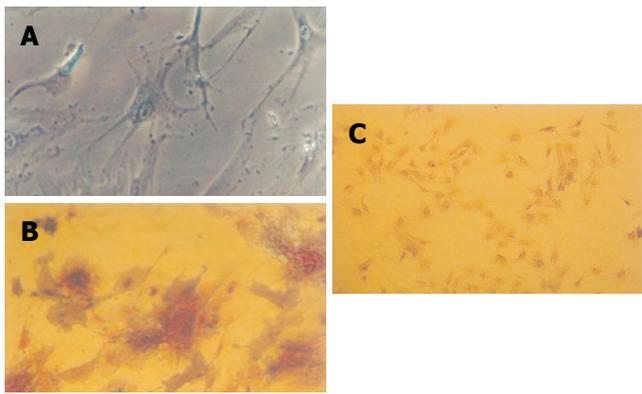


Figure 4 MSC identification. **A:** Induced by vitamin C, dexamethasone and β sodium glycerophosphate, MSC differentiated into osteoblast (200 \times); **B:** Alkaline phosphatase stain showed positive (400 \times); **C:** Alkaline phosphatase stain negative control (100 \times).

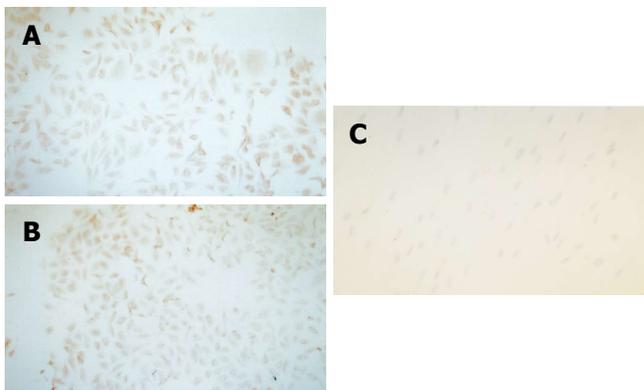


Figure 5 Expression of CK18 and AFP. **A:** Cultured with cholestatic serum for 14 d, MSC expressed AFP by S-P method (100 \times); **B:** Cultured with HGF for 14 d, MSC expressed CK18 by S-P method (100 \times); **C:** Negative control (100 \times).

manifestations could still be observed on the 35th d. As comparison, MSC cultured with HGF also showed the same distinction but the other groups did not show the feature (Figure 5).

Expression of albumin

Albumin was expressed both in MSC cultured with cholestatic serum and MSC cultured with HGF groups, but the former was more significant (Figure 6).

DISCUSSION

There are two approaches to isolate MSCs including whole-bone marrow method^[2] and density gradient centrifugation method^[3]. The former, by which we employed in this experiment, is to purify MSCs by removing the non-attached cells when culture fluid is changed, according to the different adhering capabilities of stem cell. The latter is by adherently culturing mononuclear cell, based on the conception that different cell component in myeloid has different density. Nowadays, some new techniques have been developed to isolate MSCs, such as flow cytometry and immunomagnetic beads method. However, those

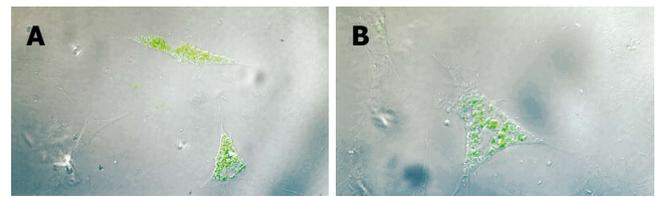


Figure 6 Expression of albumin. **A:** Culture with cholestatic serum for 35 d, expression of albumin was confirmed by in situ hybridization (FITC dyeing) (400 \times); **B:** Culture with HGF for 35 d, expression of albumin was confirmed by in situ hybridization (FITC dyeing) (400 \times).

new methods, being costly and technically difficult, have defect of inhibiting proliferation after sorting. So we preferred the whole-bone marrow method to purify MSC in this experiment, although the cells were of inadequate uniform, and heterogeneous in population.

Up to now, there has been no agreement on the phenotypical characterization of a “pure” population of human MSC despite the panoply of surface antigens reported to be expressed on MSC^[4-6]. Therefore, identification of MSC has been a puzzle. In 1999, Pittenger *et al*^[4] isolated MSC from human flank bone marrow and proved that MSC is pluripotent stem cell in that it could be induced into osteoblast, chondrocyte and lipocyte. Thus, through analyzing the differentiation phenotype during culturing, we could identify MSC. In our experiment, it was observed that osteoblast cytoplasm was basophilic and AKP stain positive. Because the osteoblast was the unique cell in differentiated MSC population, it proved the existence of MSC.

Recently, Verfaillie *et al*^[7] demonstrated that multipotent adult progenitor cells (MAPCs) from rat, mouse and human could differentiate into functional hepatocytes. They detected that the cells could synthesize urea, albumin and amylo, possess activity of cytochrome P450, adsorb α -low-density lipoprotein and did not have carcinogenesis process. It was not clear whether the MSCs we isolated included MAPCs; nevertheless, the cell could excrete CK18, AFP and albumin, which functions were similar to that of hepatocytes. AFP and CK18 are the characteristic proteins expressed during hepatocyte development. In addition, the method we used was convenient, easy to perform, not expensive and the cell still maintained cleavage and differentiation activity after numerous generations, thus it has wide application value in future.

Many investigations^[8] have revealed that bone marrow stem cell (BMSC) or purified hepatic stellate cell (HSC) can differentiate into hepatocytes. To find a suitable inducing condition has become a research hotspot lately. From the results of our study, it could be inferred that the induction of MSC by the cholestatic serum was probably through humoral signal pathway. The likely mechanism is as follows: after the choledochus was ligated, acute hepatic necrosis developed, and the liver was atrophy with a large amount of ascites in abdominal cavity; then hepatocyte growth promoters such as HGF, EGF and FGF were produced; and the cholestatic serum containing those cytokines might promote differentiation of MSC.

The concentration of cholestatic serum in our study,

50 mL/L, with coincidence to the literature^[9,10] had a good effect to induce hepatocytoid cell to secrete CK18, AFP and albumin. But the constituent of cholestatic serum, being complicated and with individual variation could not be detected precisely; therefore it needs further exploration to define ingredients of the cholestatic serum, and moreover, to verify the function of synthesis and secretion, as well as metabolism and non-carcinogenesis nature of the induced hepatocytoid cell. Bone marrow stem cells represent a safe and accessible source of stem cells, and the use of MSCs provides a potential treatment approach for severe liver diseases.

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S- Editor Wang J L- Editor Zhu LH E- Editor Ma N

RAPID COMMUNICATION

Efficacy of mycophenolate mofetil for steroid-resistant acute rejection after living donor liver transplantation

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Abstract

AIM: To discuss the use of mycophenolate mofetil (MMF) as an immunosuppressant in steroid resistant rejection after liver transplantation.

METHODS: The clinical records of 260 adult patients who underwent living donor liver transplantation (LDLT) were reviewed. Tacrolimus and methylprednisolone were used for primary immunosuppression. Acute rejection was first treated with steroids. When steroid resistance occurred, the patient was treated with a combination of steroids and MMF. Anti-T-cell monoclonal antibody was administered to patients who were not responsive to steroids in combination with MMF.

RESULTS: A total of 90 (35%) patients developed acute rejection. The median interval time from transplantation to the first episode was 15 d. Fifty-four patients were steroid resistant. Forty-four patients were treated with MMF and the remaining 10 required anti-T-cell monoclonal antibody treatment. Progression to chronic rejection was observed in one patient. Bone marrow suppression and gastrointestinal symptoms were the most common side effects associated with MMF use. There was no significant increase in opportunistic infections.

CONCLUSION: Our results demonstrate that MMF is a potent and safe immunosuppressive agent for rescue therapy in patients with acute rejection after LDLT.

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Key words: Tacrolimus; Rejection; Liver transplantation

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INTRODUCTION

In the majority of transplant centers worldwide, the standard primary immunosuppressive regimen after liver transplantation is based on calcineurin inhibitors (CNIs) and steroids^[1]. CNIs exhibit a broad spectrum of nonimmunologic side effects, including renal dysfunction, arterial hypertension, and diabetes mellitus^[2]. Despite its potent immunosuppressive effect, acute cellular and chronic rejection can still occur in patients taking CNIs, even when appropriate CNI blood trough levels are maintained^[1].

Mycophenolate mofetil (MMF), an enzyme in the guanine nucleotide synthetic pathway, inhibits the proliferation of both B and T lymphocytes^[3]. MMF is now accepted as a promising immunosuppressant for liver transplantation. Previous reports have described its efficacy as a CNI-sparing drug to reduce CNI-related toxicity in long-term survivors^[4-16]. In contrast, the role of MMF in the immediate posttransplant period is unclear^[17-19]. Here, we describe our experience using MMF for patients complicated with steroid-resistant acute rejection after living donor liver transplantation (LDLT).

MATERIALS AND METHODS

Patients

A total of 260 LDLTs (140 men and 120 women; age range: 18-67 years) were performed at the University of Tokyo Hospital between January 1996 and July 2005. The median postoperative follow-up period was 28 mo (range 1-115 mo). The most common indication was virus-related liver cirrhosis ($n = 112$) secondary to hepatitis C virus infection ($n = 78$) or hepatitis B virus infection ($n = 34$), followed by immune-mediated liver cirrhosis ($n = 74$), including primary biliary cirrhosis ($n = 56$), autoimmune hepatitis ($n = 9$), and primary sclerosing cholangitis ($n = 9$).

The range of pre-operative aspartate transaminase, total bilirubin, and serum creatinine levels were 19-308 IU/L, 4-400 mg/L, and 2-44 mg/L, respectively. The

Table 1 Target trough levels of calcineurin inhibitors and steroid dosage at Tokyo University

	Tacrolimus (ng/mL)	Cyclosporine (ng/mL)	Methylprednisolone (mg/kg per day)
POD 1-7	15-20	300-350	20-0.75
POD 8-14	14-16	250-300	0.5-0.3
POD 15-90	10-15	200-250	0.3-0.12
POD 91-180	8-10	150-200	0.08-0.12
POD 180-	5-10	100-150	0.05

POD: Postoperative day.

median score for model of end-stage liver disease was 13 (range, 4-34).

Operative and postoperative care

Our surgical technique for recipient and donor surgery is described elsewhere^[20]. All patients received tacrolimus (FK, Prograf, Astellas Pharma Inc., Tokyo, Japan) and methylprednisolone as primary immunosuppressants (Table 1). When there were FK-related adverse events^[21], FK was converted to cyclosporine A (CsA). The cytomegalovirus (CMV) status of the patient was monitored by pp65 antigenemia assay and CMV infection was defined by the presence of more than 5 antigen-positive cells/50 000 white blood cells. Fungal status was monitored by (1-3)-beta-D-glucan assay and antigen assays. Systemic fungal infection was defined as a positive polymerase chain reaction assay or positive culture with the existence of infectious foci. Systemic bacterial infection was defined as a positive culture from the bloodstream or infectious foci.

Management of rejection

Acute rejection was initially suspected by biochemical evidence of deteriorating liver function. After vascular or biliary complications were excluded, liver biopsy was performed to obtain concrete pathologic evidence of rejection. The diagnosis of acute rejection was based on internationally accepted histologic criteria^[22]. Our primary treatment for acute rejection was to administer high-dose methylprednisolone (20 mg/kg per day), followed by a gradual dose reduction with the CNI trough level around the upper range of our regimen. When there was no improvement in serum liver function tests, a second biopsy was obtained to confirm the diagnosis of steroid-resistant rejection. In these cases, oral MMF was initiated at the dosage of 3 g three times a day per mo, and then gradually tapered off within 2 to 6 mo. No reduction of CNIs and methylprednisolone was performed when the recipient was under MMF and after treatment with MMF. Anti-T-cell monoclonal antibody (OKT3, Ortho-Biotech Corporation, Raritan, NJ, USA) was used as a tertiary strategy for steroid-resistant refractory rejection under MMF and steroid recycle treatment.

Statistical analysis

Patients complicated by acute rejection were divided into

Table 2 Outcome of patients with acute rejection stratified by the rescue treatment

Group	<i>n</i>	CMV antigenemia <i>n</i> (%)	Systemic infection <i>n</i> (%)	Mortality <i>n</i> (%)
Steroid	36	14 (39)	5 (14)	7 (19)
Steroid + MMF	44	18 (41)	4 (9)	2 (5)
OKT3	10	7 (70)	4 (40)	4 (40)
Total	90	39 (43)	13 (14)	13 (14)

CMV: Cytomegalovirus; MMF: Mycophenolate mofetil.

three groups: patients treated with one-time steroid therapy ($n = 36$), those receiving MMF administration ($n = 44$), and those eventually treated with OKT3 ($n = 10$). Inter-group comparisons were performed using the chi-square test or Fisher's exact test for categorical variables. A P value of less than 0.05 was considered statistically significant.

RESULTS

Outcome

A total of 90 out of 260 patients developed acute rejection (35%, 90/260). The median interval time from transplantation to the primary episode of acute cellular rejection was 15 d (range 5-637 d). Fifty-four patients presented with steroid-resistant rejection and were treated with a second steroid recycle in combination with MMF. The median duration of MMF administration in these 54 patients was 74 d (range 36-182 d). Of the 54 patients who received MMF, 10 had refractory acute rejection requiring the use of OKT3. The median interval between the addition of MMF and the use of OKT3 was 5 d (range 2-8 d). Among the patients treated with OKT3, two required the additional use of basiliximab (Simulect, Novartis Pharma, Tokyo, Japan). Chronic rejection was observed in one patient (0.04%, 1/260) who eventually required re-transplantation. Graft failure due to uncontrollable acute rejection was experienced in one patient (0.04%, 1/260) who died 49 d after LDLT, despite the combined use of MMF, OKT3, and basiliximab.

Outcome stratified by treatment

Mortality and systemic bacterial/fungal infections were significantly higher in the patients treated with OKT3 than in the other groups ($P = 0.02$ and 0.04 , respectively). The incidence of positive CMV antigenemia tended to be higher in the patients treated with OKT3, although the difference was not statistically significant (Table 2).

Side effects of MMF

MMF-associated side effects were observed in 11 patients (20%), bone marrow suppression in 9 patients (17%), and gastrointestinal symptoms in 2 patients (4%). A dose reduction of MMF and granulocyte colony stimulating factor administration was sufficient for all the patients with bone marrow suppression. Gastrointestinal symptoms disappeared spontaneously under the use of MMF. Cessation of MMF was not necessary due to adverse effects.

DISCUSSION

The results of our study together with those of other studies^[17,19] demonstrate that MMF can influence the course of steroid-resistant acute rejection. The main advantage of MMF rescue therapy is the option of continuing the therapy^[19]. MMF therapy can be continued in selected patients on an outpatient basis. Rejection rescue therapy with OKT3, anti-thymocyte globulins, and anti-lymphocyte globulin, in contrast, permits only limited use for a short period of time.

Another advantage of MMF is that adverse events related to MMF are infrequent and often mild, which allows for long-term administration when required. In our series, bone marrow suppression and gastrointestinal symptoms were the most common adverse events of MMF. These episodes were easily reversed by dose reduction. MMF was not associated with a significantly increased risk of opportunistic infections. These results are compatible with previous reports^[5,7,11].

LDLT theoretically offers an immunologic advantage when the donors are related to the recipients^[23]. The overall incidence of acute rejection, however, is similar between LDLT and deceased donor liver transplantation. Our series demonstrated that the overall incidence of steroid-resistant acute rejection was 21%, which was unexpectedly high because LDLT recipients have been reported less likely to develop steroid-resistant or chronic rejection^[24]. The 'immunologic advantage' of LDLT might be smaller than previously expected.

In conclusion, the results of our retrospective study suggest that treatment with MMF might be indicated for selected patients with acute rejection and demonstrate the high clinical value of MMF for secondary immunosuppressive therapy after LDLT.

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Comparing mass screening techniques for gastric cancer in Japan

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Abstract

AIM: To discuss the efficacy of endoscopic mass screening for gastric cancer.

METHODS: The data used in this study were the results of mass screening programs for gastric cancer in Niigata City from 2002 to 2004. The number of participants was 35089 in 2002, 34557 in 2003 and 36600 in 2004. The finding ratio referred to the final diagnosis of gastric cancer after a double check of endoscopic files and histological findings. The costs of identifying one case of gastric cancer were calculated based on the total expense for each screening program and additional close examinations.

RESULTS: From the analysis of individual screening program with endoscopy, individual screening program with X-ray (ISX) and mass screening program with photofluorography (MSP) in reference to the finding ratio of gastric cancer, endoscopic examination was the best for detecting early gastric cancer, the finding ratio was 0.87% in 2004, approximately 2.7 and 4.6 times higher than those of the ISX and MSP groups. In addition, this novel method was the cheapest means regarding the cost of identifying one case of gastric cancer, which was estimated to be 1608000 Japanese yen in 2004.

CONCLUSION: Endoscopic mass screening is a promising method and can be effectively applied if a sufficient number of skilled endoscopists become available to staff the system and if city offices support it.

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Key words: Mass screening; Gastric cancer; Endoscopy; Cost effectiveness

INTRODUCTION

Gastric cancer is recognized worldwide as the second leading cause of cancer mortality^[1]. In Japan, non-cardia gastric cancer still remains the major cause of cancer death, although recently the mortality rate has begun to decrease^[2]. With reference to this change, it has been reported that early detection of gastric cancer may contribute to decreases in the mortality rate^[3,4].

Japan has carried out ongoing programs to prevent gastric cancer death by mass X-ray screening methods to detect gastric cancer in its early stages^[4,5]. In recent years, endoscopy has been applied instead of X-ray as the initial mass screening method in several cities in Japan, and endoscopic mass screening has been utilized in Niigata City since 2003. The efficacy of endoscopic mass screening is discussed herein.

It is known that the finding ratio of early gastric cancer with endoscopy is higher than that with X-ray^[6], making endoscopy a highly effective screening method^[7]. There are, however, cost problems related to applying endoscopic mass screening^[6,7]. Endoscopic screening is more expensive than direct X-ray screening (ordinary upper GI) in the Japanese medical system. Although the balance is approximately 3400 Japanese yen, because of financial problems in city offices, at the start of the present study, the costs for the two screening procedures were decided to be the same for a while.

MATERIALS AND METHODS

Subjects

The data used in this study were the results of mass screening programs for gastric cancer in Niigata City from 2002 to 2004. The number of participants was 35089 in 2002, 34557 in 2003 and 36600 in 2004. As employees have their own mass screening system of Japan, nonemployees participated in these mass screenings. All screening participants aged 40 or over could choose one of the three programs utilizing endoscopy (ISE), direct X-ray (ISX) and photofluorography (MSP: indirect X-ray

Table 1 Transition before and after adoption of endoscopic screening program

		2002	2003	2004
Participants (n)	ISE	-	8118	11 679
	ISX	28 332	20 058	19 011
	MSP	6757	6381	5910
Detection rate (%)	ISE	-	0.81	0.87
	ISX	0.33	0.31	0.32
	MSP	0.25	0.22	0.19
Rate of early gastric cancer (%)	ISE	-	74.2	75.5
	ISX	40.8	56.5	63.9
	MSP	46.7	71.4	54.5
Cost (J YEN)	ISE	-	1 693 000	1 608 000
	ISE ²		2 113 000	1 998 000
	ISX	5 113 000	4 365 000	4 177 000
	MSP	2 712 000	2 792 000	3 290 000

ISE: Individual screening program with endoscopy; ISE²: Cost analysis with ordinary endoscopic examination fee; ISX: Individual screening program with X-ray; MSP: Mass screening program with photofluorography; Cost: Total cost of finding one gastric cancer patient.

with a small sized film) each.

Analysis

The finding ratio referred to the final diagnosis of gastric cancer after a double check of endoscopic files and histological findings. The costs of identifying one case of gastric cancer with each method were calculated based on the total expense for each screening program and additional close examinations.

Every citizen participates in some type of a medical insurance program, which enables all Japanese have access to medical services for next close examinations at the same costs. X-ray examination of suspected gastric cancer in ISX and MSP groups showed that the patients with suspected lesion could be referred to the endoscopists to receive endoscopy with biopsy for getting a final histological diagnosis. We included these close examination fees in addition to the screening fee for cost analysis. In ISE group the costs of endoscopic screening and additional biopsy at the same time (if needed) were also counted.

RESULTS

Finding ratio

In 2004, the finding ratio in the ISE group was 0.87%, approximately 2.7 and 4.6 times higher than those of the ISX and MSP groups respectively and the rate of early gastric cancer in the ISE group was the highest among the three groups.

Cost analysis

The cost of identifying one case of gastric cancer was estimated to be 1 608 000 Japanese yen in ISE, 4 177 000 Japanese yen in ISX, and 3 290 000 Japanese yen in MSP in 2004. In the same condition of ordinary medical fee for endoscopic examination, the cost of ISE increased to 1 998 000 Japanese yen.

These results indicated that endoscopic mass screening was the best and cheapest means for detecting and identifying gastric cancer. The results of each mass screening are shown in Table 1.

DISCUSSION

To decrease cancer death, early detection and subsequent surveillance are necessary^[8]. Although eradication of *H pylori* infection is the most effective, the incidence of gastric cancer would remain high for several decades to come^[9]. Endoscopic mass screening for gastric cancer is effective in identifying cancer in its early stages^[6], but endoscopic screening is more expensive than direct X-ray screening in the Japanese medical system. In this study our cost analysis showed that even if we applied ordinary examination fee for endoscopic mass screening, this novel approach was still the most superior method in cost-effectiveness for finding gastric cancer patients.

On the other hand, endoscopic mass screening is practically not appropriate for wide application due to the limited number of skilled endoscopists^[6]. In Niigata City, however, we fortunately have enough endoscopists who have the title of authorized specialists as a board-certified member to examine more than 10 000 cases yearly, making it feasible to adopt an endoscopic screening system.

In mass screening systems for gastric cancer, endoscopic examination is a promising method and can be effectively applied if a sufficient number of skilled endoscopists become available to staff the system and if city offices support it.

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Factors predicting poor prognosis in ischemic colitis

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Abstract

AIM: To determine the clinical, analytical and endoscopic factors related to ischemic colitis (IC) severity.

METHODS: A total of 85 patients were enrolled in a retrospective study from January 1996 to May 2004. There were 53 females and 32 males (age 74.6 ± 9.4 years, range 45-89 years). The patients were diagnosed as IC. The following variables were analyzed including age, sex, period of time from the appearance of symptoms to admission, medical history, medication, stool frequency, clinical symptoms and signs, blood tests (hemogram and basic biochemical profile), and endoscopic findings. Patients were divided in mild IC group and severe IC group (surgery and/or death). Qualitative variables were analyzed using chi-square test and parametric data were analyzed using Student's *t* test ($P < 0.05$).

RESULTS: The mild IC group was consisted of 69 patients (42 females and 27 males, average age 74.7 ± 12.4 years). The severe IC group was composed of 16 patients (11 females and 5 males, average age of 73.8 ± 12.4 years). One patient died because of failure of medical treatment (no surgery), 15 patients underwent surgery (6 after endoscopic diagnosis and 9 after peroperative diagnosis). Eight of 85 patients (9.6%) died and the others were followed up as out-patients for 9.6 ± 3.5 mo. Demographic data, medical history, medication and stool frequency were similar in both groups ($P > 0.05$). Seriously ill patients had less hematochezia than slightly ill patients (37.5% vs 86.9%, $P = 0.000$). More tachycardia (45.4% vs 10.1%, $P = 0.011$) and a higher prevalence of peritonism signs (75% vs 5.7%, $P = 0.000$) were observed in the severe IC group while the presence and intensity of abdominal pain were similar between two groups. Two patients with severe IC had shock when admitted. Regarding analytical data, more seriously ill patients were found to have anemia and hyponatremia

than the mildly ill patients (37.5% vs 10.1%, $P = 0.014$ and 46.6% vs 14.9%, $P = 0.012$, respectively). Stenosis was the only endoscopic finding that appeared more frequently in seriously ill patients than in slightly ill patients (66.6% vs 17.3%, $P = 0.017$).

CONCLUSION: The factors that can predict poor prognosis of IC are the absence of hematochezia, tachycardia and peritonism, anemia and hyponatremia and stenosis.

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Key words: Ischemic colitis; Hematochezia; Tachycardia; Peritonism; Anemia; Hyponatremia ; Stenosis

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INTRODUCTION

Ischemic colitis (IC) is the most frequent form of vascular alterations in the digestive system. It occurs mainly in the elderly with pluripathology when there is no major vascular occlusion as a result of reduced blood flow responsible for the colon's needs, and is conditioned by many factors^[1].

Clinical presentations vary from mild and limited forms not needing medical treatment to fulminant trans-mural colonic necrosis which may lead to death of patients. The variability in presentations of IC makes epidemiologic research difficult in the general population. The most frequently observed symptoms are abdominal pain and hematochezia^[2].

If IC is clinically suspected, its diagnosis can be established by colonoscopy. The most frequent locations are the splenic flexure, the descending colon and the sigmoid colon, although any segment of the colon can be affected^[3].

Evolution of IC in patients depends on the severity of the presentation. Most mildly affected patients are asymptomatic after medical treatment. Severely affected patients and those that have strictures need to undergo surgery and have a higher morbidity and mortality rate.

The possibility of establishing prognostic factors promptly is of great importance in deciding the best therapeutic strategy for each case. However, only a few

studies on the possible relation of etiological, pathogenic and clinical factors with the progression to colitis are available^[1,3-11].

This study was to review the clinical, biological and endoscopic data of ischemic colitis patients admitted to our hospital and to discover the prognostic factors that determine the evolution of ischemic colitis.

MATERIALS AND METHODS

Patients

A total of 85 patients who were admitted to our hospital from January 1996 to May 2004 and diagnosed with ischemic colitis were retrospectively analyzed. The diagnosis was suspected based on the clinical and X-ray findings and confirmed by the endoscopic and histologic results or the exploratory laparotomy findings. The patients with ischemic colitis secondary to vascular surgery, thromboembolism of the mesenteric artery and/or rectocolonic tumour were excluded.

Methods

The clinical variables including age, sex, period of time from the onset of symptoms to admission and colonoscopy, personal medical history (especially cardiovascular and metabolic history), medication taken regularly, previous stool frequency, symptoms at onset (general well being, abdominal pain, and hematochezia) were analyzed. The exploration signs on admission including hemodynamic state, especially peripheral collapse (systolic blood pressure < 90 mmHg with signs of decreased peripheral blood flow and high blood pressure (systolic blood pressure > 140 mmHg and/or diastolic blood pressure > 90 mmHg) and abdominal exploration, mainly signs of peritoneal irritation such as rigidity and rebound tenderness (Blumberg's sign) were evaluated.

The usual biological, hematological and biochemical parameters obtained on admission were also analyzed. The normal ranges of values provided by our hospital laboratory were used for the qualitative analysis of the main blood test parameters (basic hemogram and biochemical markers).

The endoscopic and laparoscopic results were described identifying the segment of the affected colon. The colon was divided into ascending colon (superior mesenteric artery) and descending colon (inferior mesenteric artery) according to the artery responsible for each segment, sigmoid and descending colon, transverse colon and ascending colon according to the location.

Patients were divided in two groups: severe ischemic colitis patient group (who needed surgery or died in the episode), and mild ischemic colitis patient group (good evolution with medical treatment). Medical treatment included fasting, symptomatic treatment, hydro-electrolytic correction and support, and use of wide spectrum antibiotics.

Statistical analysis

Data were introduced and analyzed using SPSS version 11.0. Qualitative variables were analyzed with the chi-

square test (Fisher's correction when necessary) and the parametric data were analyzed with the Student *t* test for independent groups. Variables with $P < 0.1$ in the univariate analysis were submitted to logistic regression analysis. $P < 0.05$ was considered statistically significant.

RESULTS

Patients

The study included 85 patients (53 women and 32 men) with an average age of 74.6 ± 9.4 years (range 39-89 years). The main risk factor was high blood pressure which was present in 55 patients (64.7%), followed by other cardiovascular diseases (37.6%), diabetes (29.4%), chronic obstructive pulmonary disease (11.8%) and chronic renal insufficiency (1.2%). Previous cardiovascular illness was confirmed in 80% of the patients. Medications taken during the 15 d before the diagnosis of ischemic colitis were registered: antihypertensive medication (64.7%), nonsteroidal antiinflammatory drugs (NSAID) (35.3%), diuretics (25.9%), anticoagulants (3.5%) and cardiotoxic drugs (2.4%). Chronic constipation was confirmed in 52 out of 71 patients (73.2%), while regular laxative intake was verified in 39 out of 66 patients (59.1%). The main symptoms that motivated medical attendance were abdominal pain (72/85 patients, 84.7%) and hematochezia (66/85 patients, 77.6%). Immediate physical exploration revealed that two patients suffered from shock at admission (systolic blood pressure < 90 mmHg), while 47.5% of the patients had high blood pressure. Abdominal exploration was compatible with peritonism (positive Blumberg's sign) in 16 cases (18.8%).

The diagnosis was established by endoscopy with biopsies (73 patients) and surgery (12 patients). A conventional abdominal ultrasound was performed in 43 patients (50.6%) and a CT-scan was done in 19 patients (22.4%) prior to endoscopy and/or surgery.

The lesions were distributed depending on their locations: descending colon in 66 patients (77.6%), splenic angle in 10 patients (11.8%), transverse colon in 4 patients (4.7%) and ascending colon in 4 patients (4.7%).

Mild ischemic colitis was found in 69 patients (81.2%) including 27 men and 42 women with an average age of 74.7 ± 8.6 years (range 45-89 years). All the patients improved after medical treatment. The severe ischemic colitis patient group was consisted of 16 patients (18.82%) including 5 men and 11 women, with an average age of 73.8 ± 12.4 years (range 39-85 years). One patient died due to failure of the medical treatment, while 15 patients underwent surgery (6 patients who were diagnosed by colonoscopy deteriorated despite medical treatment and 9 patients were directly diagnosed during surgery). Eight patients (9.6%) died. The rest of the patients were followed up at the out-patient clinic for an average period of 9.6 ± 3.5 mo (1-72 mo).

Severity prediction factors

The comparative analysis of mild and severe IC showed no statistically significant differences with respect to age, sex, past medical history, previous medication, stay in hospital,

Table 1 Clinical characteristics of the patients with ischemic colitis

	Total <i>n</i> = 85	Mild <i>n</i> = 69	Severe <i>n</i> = 16
Age (yr)	74.6 ± 9.4	74.7 ± 8.6	73.8 ± 12.4
Sex (M/F)	32/53	27/42	5/11
Hospital stay (d)	8.5 ± 6.8	8.0 ± 5.5	10.4 ± 10.9
HBP, <i>n</i> (%)	55 (64.7)	48 (69.6)	7 (43.8)
Diabetes, <i>n</i> (%)	25 (29.4)	20 (29)	5 (31.3)
Cardiovascular illness, <i>n</i> (%)	32 (37.6)	26 (37.7)	6 (37.5)
CRF, <i>n</i> (%)	1 (1.2)	1 (1.4)	0 (0)
COPD, <i>n</i> (%)	10 (11.8)	6 (8.7)	4 (25)
Constipation, <i>n</i> (%)	52 (61.2)	42 (60.9)	10 (62.5)
Hematochezia, <i>n</i> (%)	66 (77.6)	60 (87)	6 (37.5) ^b
Abdominal pain, <i>n</i> (%)	72 (84.7)	57 (82.6)	15 (93.8)
Peritonism, <i>n</i> (%)	16 (18.8)	4 (5.8)	12 (75) ^b
Systolic BP (mmHg)	142.01 ± 28.3	145.2 ± 28.8	128.4 ± 22.2
Diastolic BP (mmHg)	77.7 ± 13	79.2 ± 13.3	71.2 ± 9.5
Heart rate	82.2 ± 16.2	80.1 ± 13.9	93.5 ± 22.3 ^a
Anemia	13	7	6 ^a
Leukocytosis (> 108 × 10 ⁹ /L)	73	60	13
Uremia	26	23	3
Hyponatremia	17	10	7 ^a

^a*P* < 0.05, ^b*P* < 0.01. Hronic renal failure, COPD: Chronic obstructive pulmonary disease.

stool frequency and location of the lesion in the intestine (Table 1).

Regarding the clinical presentation of the illness, statistically significant differences were observed between the two groups. Hematochezia was most frequently seen in the mild IC patient group compared to the severe IC patient group (86.9% *vs* 37.5%), while signs of peritonism were mostly found in the severe IC patient group compared to the mild IC patient group (75% *vs* 5.7%, *P* < 0.001). No differences were observed in the presence and intensity of abdominal pain (Table 1).

On admission, tachycardia (heart rate > 90 b/min) was found most frequently in the severe IC patient group compared to the mild IC patient group (45.4% *vs* 10.1%, *P* < 0.008). The average blood pressure (systolic and diastolic) was higher in the mild IC patient group than in the severe IC patient group (145.2 ± 28.8 mmHg and 79.2 ± 13.3 mmHg *vs* 128.4 ± 22.2 mmHg and 71.2 ± 9.5 mmHg, *P* < 0.05). However, the number of patients with high blood pressure was similar in both groups. The two patients suffering from shock at admission had severe IC (Table 1).

Biological parameter analysis showed that there were statistically significant differences between the two groups in haemoglobin (normal 120-160 g/L), platelet (normal 130 × 10⁹/L-400 × 10⁹/L) and glycemia (3.9-6.2 mmol/L) values, and similar results in the rest of the parameters (Table 1). Patients with severe IC had anemia most frequently (haemoglobin < 120 g/L) when compared to patients with mild IC (37.5% *vs* 10.1%, *P* = 0.012) and hyponatremia (serum sodium < 136 mmol/L) (46.6% of the severe *vs* 14.9% of the mild, *P* = 0.012).

No statistically significant differences were observed with respect to the endoscopic lesions (erythema, edemas, hematomas, ulcers and fibrin deposits) except for the strictures which were more frequent in the severe IC patient group (66.6% *vs* 17.3%, *P* = 0.017).

Logistic regression analysis showed that hematochezia was a weak predictor of mild IC (OR = 0.09, IC95% = 0.026-0.308, *P* = 0.000), while signs of peritonism (OR = 48.7, IC95% = 10.6-222.1, *P* = 0.000), tachycardia (OR = 7.36, IC95% = 1.71-31.5, *P* = 0.007), anemia (OR = 5.31, IC95% = 1.47-19.08, *P* = 0.010) and hyponatremia (OR = 4.98, IC95% = 1.47-16.8, *P* = 0.010) were associated with severe IC.

DISCUSSION

Ischemic colitis is a well defined illness that is frequently found in elderly patients and is generally associated with clinical or therapeutic situations. The true incidence in the general population or in groups of patients with specific illnesses is not well known because it depends both on the ability of physicians to suspect the diagnosis and on the thoroughness of the diagnostic process. It was reported that the incidence is 6.1 to 47 in 100000 inhabitants/year^[12]. No systematic studies have established the association between ischemic colitis and cardiovascular pathology, chronic obstructive pulmonary disease, use of different medications and all circumstances responsible for a reduction in blood flow. Our patients had a high prevalence of these factors.

Observational and meta-analysis studies that point to a relationship between IC and irritable bowel syndrome and their treatment with antagonists of 5-HT₃ receptors have recently been published^[13-16]. Whether they are different stages of the same process remains unclear. Treatment sequelae or the final consequence of an altered phenomenon that was previously considered functional has to be explained.

Another predisposing factor for IC may be constipation, probably due to the increased colonic luminal pressure which is responsible for worse blood flow in the colonic wall. All these factors, in addition to the progressive aging of the population, make us presume a future increase in its incidence. The diagnostic and therapeutic strategies need to be further studied.

Clinical presentation in ischemic colitis varies from mild forms that heal after medical treatment to severe forms with complete necrosis of the colonic wall requiring surgery and/or leading to death of patients. In general, the first symptoms of IC are hematochezia and abdominal pain as confirmed in our series.

Clinical, biological and/or endoscopic factors capable of promptly discriminating both groups of patients can be used to take the most appropriate therapeutic steps. Few studies have evaluated this aspect of IC^[8-11].

Out of all the clinical parameters evaluated in our patients, that standing out as a possible predictor for the evolution of IC is only hematochezia. As a clinical presentation, it can protect the patient from severe IC, because in our study it was significantly more frequent

in the mild forms. The presence of tachycardia and peritonism on physical exploration (in our study significantly more frequent in the severe forms of IC) is possibly an expression of the hemodynamic instability and the colonic trans-mural injury, which could lead to perforation and subsequent peritonitis.

The only biological parameters are anemia and hyponatremia which were more frequent in severe forms of IC in our study.

In our series of patients, we did not find a relationship between the evolution of IC and the anatomical location of the lesions, regardless of the diagnostic method applied (endoscopy or surgery), which is different from that disclosed by other authors^[9].

In this study, we found no relationship between age, sex and the subsequent evolution of IC as in other studies^[8,9]. Pla *et al*^[11] and Barouk *et al*^[10] reported that they have established a worse prognosis in elderly patients. There are also incongruent results with regard to the relationship of the medical history of patients and the severity of IC. Medina *et al*^[9] reported that there is no relationship, while others^[8,10-11] reported that high blood pressure is a predisposing factor for the worse evolution of the illness.

The mortality of our patients was almost 10% and all the patients suffered from severe IC, differing greatly from that (66%) reported in the literature^[17].

In conclusion, ischemic colitis is an illness that should be considered in any elderly patients with clinical and therapeutic risk factors, such as abdominal pain and/or hematochezia. The mortality rate of ischemic colitis is still high. We hold that it is necessary to carry out prospective and controlled studies in order to clearly detect the factors that predict the evolution form and find the best therapeutic approach.

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Histopathological comparison of topical therapy modalities for acute radiation proctitis in an experimental rat model

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Formalin; Betamethasone; Misoprostol

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Abstract

AIM: To evaluate the prevalent topical therapeutic modalities available for the treatment of acute radiation proctitis compared to formalin.

METHODS: A total of 120 rats were used. Four groups ($n = 30$) were analyzed with one group for each of the following applied therapy modalities: control, mesalazine, formalin, betamethasone, and misoprostol. A single fraction of 17.5 Gy was delivered to each rat. The rats in control group rats were given saline, and the rats in the other three groups received appropriate enemas twice a day beginning on the first day after the irradiation until the day of euthanasia. On d 5, 10, and 15, ten rats from each group were euthanized and a pathologist who was unaware of treatment assignment examined the rectums using a scoring system.

RESULTS: The histopathologic scores for surface epithelium, glands (crypts) and lamina propria stroma of the rectums reached their maximum level on d 10. The control and formalin groups had the highest and mesalazine had the lowest, respectively on d 10. On the 15th d, mesalazine, betamethasone, and misoprostol had the lowest scores of betamethasone.

CONCLUSION: Mesalazine, betamethasone, and misoprostol are the best topical agents for radiation proctitis and formalin has an inflammatory effect and should not be used.

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Key words: Acute radiation proctitis; Mesalazine;

INTRODUCTION

Acute radiation proctitis is a major clinical complication of pelvic irradiation^[1,2]. Rectum is exposed to high dose irradiation during radiotherapy of the rectal, cervical and prostate malignancies. The rate of complications, which decrease life quality, has been reported as high as 10%-15%^[3,4].

The problem of radiation proctitis leads to prophylaxis studies. Sukralfat^[5-9], anti-inflammatory agents^[6,9], mesalazine^[5] have been shown to be histopathological ineffective in clinical prophylaxis studies. In spite of the clinical comparative studies of these drugs there is no agreement on the therapy protocol^[8,10-12].

There are several therapy modalities used for the protection and treatment of acute radiation proctitis and consequent chronic sequelae, including diet^[13], sucralfate^[14,15], antiinflammatory agents like mesalazine^[15,16], corticosteroids^[5,10], formalin^[17,18] and misoprostol^[13,6]. However these medical approaches are not sufficiently effective and have only a limited benefit. The search for a more effective therapy should be continued.

The pathological features of irradiation begin in hours. Morphologic changes include loss of columnar shape, and nuclear polarity of enterocytes, epithelial degeneration, ulceration, nuclear pyknosis and karyorrhexis, crypt disintegration, mucosal edema, absent mitosis, crypt abscesses with prominent eosinophilic infiltrates^[19].

The aim of this study was to compare the most prevalent topical therapeutic modalities used for acute radiation proctitis in a standard rat radiation proctitis model^[20].

MATERIALS AND METHODS

Animals

Female Sprague Dawley rats weighing 230-285 g at the age of 6 wk were obtained from the Institute for Experimental Medicine, Istanbul University. All animals were acclimatized for 7 d prior to experimentation in the laboratory of the

Table 1 Grading of histological changes on microscopic examination

Structure	Characteristic
Surface epithelium	Loss of cellular height/flattening of cells Cellular inflammatory infiltrate
Glands (crypts)	Luminal migration of epithelial nuclei Loss of goblet cells Mitotic activity Cryptitis (migration of segmental neutrophils through the crypt wall) Eosinophilic crypt abscesses Loss of glands Atrophy of glands Distortion of glands
Lamina propria	Inflammation Edema Congestion of blood vessels

The abnormalities were assessed as normal (score = 0) or abnormal, ranked according to severity and arranged in quartiles.

mentioned institute, which was maintained at $22 \pm 2^\circ\text{C}$ and a relative humidity of $55 \pm 10\%$ in a constant 12 h dark/light cycle. The rats were housed in standard wire cages and fed with standard rodent chow and UV sterilized tap water. The approval of the Ethical Review Committee of the Faculty was obtained and the experimental procedures in this study adhered to the Declaration of Helsinki for the care and use of laboratory animals.

After irradiation each animal was weighed and examined every 2 d until the end of the experiment. Five groups (30 rats in each group) were used. Groups were defined as follows. Rats in control group were anesthetized, restrained and taped by the tail and legs on an acryl plate, irradiated and given saline enemas. Rats in mesalazine group were irradiated, and given mesalazine enemas (Salofalk, Ali Raif, Istanbul, Turkey)^[21]. Rats in formalin group were irradiated, and given formalin enemas (4%)^[22]. Rats in betamethasone group were irradiated, and given betamethasone enemas (Betnesol, Glaxo-Wellcome, Hamburg, Germany)^[13]. Rats in isoprostol group were irradiated, and given misoprostol enemas (5 $\mu\text{g}/\text{kg}$)^[23]. The enemas, which were prepared in 1 mL volume at body temperature, were given twice a day beginning on the first day after irradiation until the euthanasia day. The enemas were applied with a soft feeding tube and then the anus was temporarily closed with digital compress for five minutes.

Irradiation

Each rat was anesthetized using sodium pentobarbital intraperitoneally (40 mg/kg). Then 3 to 4 rats at a time were restrained and taped by the tail and legs on an acryl plate at a supine position. Lead shielding (5 half value layer) was used to cover the rat except for a 3 cm \times 4 cm area of lower pelvis containing 2 cm length of rectum in the middle of the field. Irradiation was delivered with a cobalt-60 apparatus, the γ energy was 1.25 MeV, and a distance of 80 cm from the source to the surface was used. Dose rate of the irradiation was 121.49 Gy per min and 17.5 Gy in a single fraction was delivered to each rat. Ten rats from

Table 2 Comparison of the histopathological scores of the rectal specimens with regard to the euthanasia days (mean \pm SD)

	5 th d	10 th d	15 th d
Control	7.40 \pm 3.89	39.20 \pm 3.58 ¹	35.40 \pm 2.27
Mesalazine	5.60 \pm 3.41	11.40 \pm 3.37	22.40 \pm 2.37
Formalin	10.0 \pm 4.50	38.20 \pm 3.61 ²	37.00 \pm 2.26 ²
Betamethasone	3.20 \pm 1.03	20.60 \pm 3.41	18.20 \pm 4.32
Misoprostol	4.40 \pm 1.71	20.80 \pm 2.70	23.6 \pm 2.80 ²

¹ Two specimens were excluded; ² One specimen was excluded.

each group were selected randomly for gross and macroscopic examination and euthanized on d 5, 10, and 15. Two 5 mm segments of the rectum which was 1 cm proximal to anus were excised, fixed in 10% neutral buffered formalin solution and processed by routine techniques. Each specimen was stained with hematoxylin and eosin and examined under a light microscope by a pathologist who was unaware of treatment assignment.

The rectums were evaluated microscopically using a slightly modified scoring system reported by Hovdenak *et al*^[7] by a pathologist who was blinded to the groups and the euthanasia days of specimens. A total of 13 characteristics of three mucosal structures (surface epithelium, glands, and lamina propria stroma) were used. The abnormalities of the 13 parameters were assessed as normal (score = 0) or abnormal, and ranked according to severity and arranged in quartiles: 1 = mildly abnormal; 2 = moderately abnormal; 3 = markedly abnormal; 4 = severely abnormal.

Statistical analysis

Results were presented as mean \pm SD. Relationship between the groups was assessed using analysis of variance. Tukey or Tamhane's test was chosen to test for equality of variances. *P* values less than 0.05 were considered statistically significant.

RESULTS

There was no death during the study. For determination of the acute radiation injury and the response to the local therapy, 10 rats from each group were euthanized on d 5, 10 and 15 after the irradiation. The rats were apparently healthy until the day of sacrifice excluding three rats (2 in control group and 1 in formalin group), which "looked" ill and showed symptoms of diarrhea on the 10th d. The rectums of the rats were removed and examined grossly and histopathologically. Tissues from five specimens (including the three mentioned above) had severe damage and were inappropriate to be scored. They were thus excluded from the study.

The five samples excluded were two from control group on the 10th d due to the ischemic necrosis of the rectal mucosa, one from formalin group on the 10th d due to massive mucosal infarct, one from formalin group on the 15th d due to lymphocytic colitis, one from isoprostol group on the 15th d due to lymphoid hyperplasia and submucosal edema (Table 1).

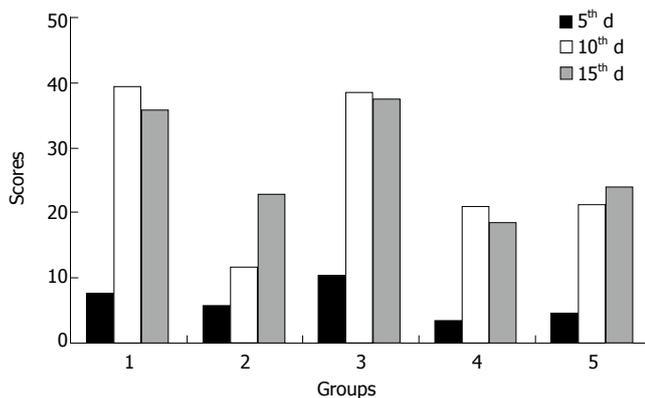


Figure 1 Sum of the histopathologic scores regard to the euthanasia days. 1: control group; 2: mesalazine group; 3: formalin group; 4: betamethasone group; 5: misoprostol group. The differences in the mean scores of all groups, but the formalin group on the 10th and 15th d and betamethasone group on the 10th and 15th d were statistically significant.

The mean histopathologic scores of the specimens are shown in Table 2.

On the 5th d the formalin group had the highest scores, followed by the control group. Mesalazine, betamethasone and misoprostol groups had the lowest scores. Table 2 gives the statistical comparison of the scores. There was no difference between control and formalin groups. The mesalazine, betamethasone and misoprostol groups had no difference between each other either. The control group had significantly higher scores than betamethasone group ($P = 0.039$). The formalin group had higher scores than the mesalazine, betamethasone and misoprostol groups ($P = 0.028, 0.0001$ and 0.003 , respectively).

On the 10th d the control and formalin groups had the highest scores, followed by misoprostol, betamethasone and mesalazine groups (Table 2). The scores of the control and formalin groups were higher than those in the misoprostol, betamethasone and mesalazine groups ($P = 0.0001$). There was no difference between control and formalin groups. The mesalazine group had the significantly lowest scores ($P = 0.0001$). There was no statistically significant difference between misoprostol and betamethasone groups.

On the 15th d, the scores of formalin and control groups were the highest (Table 2). Misoprostol, mesalazine and betamethasone groups had lower scores. The formalin and control groups had significantly higher scores than the other groups ($P = 0.0001$). There was no difference between mesalazine and misoprostol groups. The betamethasone group had the lowest scores ($P = 0.0001$ for the control and formalin groups, and $P = 0.044$ for the misoprostol group).

The sum of the histopathologic scores on the euthanasia days are presented on Figure 1. The inflammatory processes of the control, formalin and betamethasone groups reached a maximum score on the 10th d and decreased on the 15th d. Since inflammation of the mesalazine and misoprostol groups increased on the 10th and 15th d, the scores of the last group were higher. The mean scores of all groups, except for the formalin group and the beta-

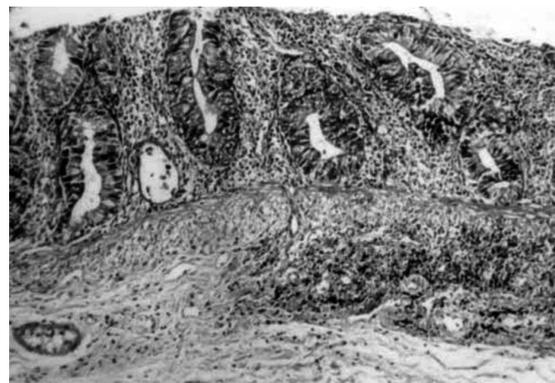


Figure 2 Cryptitis, loss of cellular height/flattening of cells, nuclear debris in the crypt lumen and mononuclear leukocyte infiltration on lamina propria (HE X 125).

methasone group on the 10th and 15th d, were significantly different.

DISCUSSION

The aim of this study was to compare the histopathologic effects of formalin therapy with the most common agents used for radiation proctitis^[4,12,18,24-26]. The consequential correlation between acute injury, which is common and usually self-limiting and chronic sequela has been demonstrated^[27,28].

With a controlled radiation proctitis animal model, the four most widely used therapeutic approaches were studied in a blinded histopathologic comparison. This standard model has two handicaps: the impossibility to examine the same individual over the time and the relation of the pathologic scores to the clinical symptoms. To deal with these problems, we used groups of simultaneously sacrificed rats and a pathological scoring system obtained from a clinical study^[7]. The chosen model was justified with comparison to the clinical series from the pathological point of view in regard to the duration after irradiation and the optimal dose for the human disease. The protective dose of various medications has been determined to be 17.5 Gy in single fraction^[20,29].

The acute pathological findings on the rectal mucosa are reported to persist for two weeks^[20,29]. An edema of the lamina propria was observed in a first couple of days, cryptitis and crypt abscesses were found on d 4 and 5, ulcers and regenerative processes could be determined on d 9 and 10, the mucosa was healed and then the chronic sequelae like fibrosis of the lamina propria arose on d 15^[7].

It was reported that the pathological differences in the 10th d groups are more obvious^[7]. Cryptitis (Figure 2) and crypt abscesses (Figure 3) are the predominant characteristics of acute radiation toxicity. Two specimens of the control group showed mucosal ischemic necrosis. Two specimens of the formalin group showed mucosal infarct. One specimen of the misoprostol group showed crypt hyperplasia. The mean and sum mucosal and glandular pathologic scores of the formalin group were close to those of the control group. Betamethasone and misoprostol groups had statistically significant low scores.

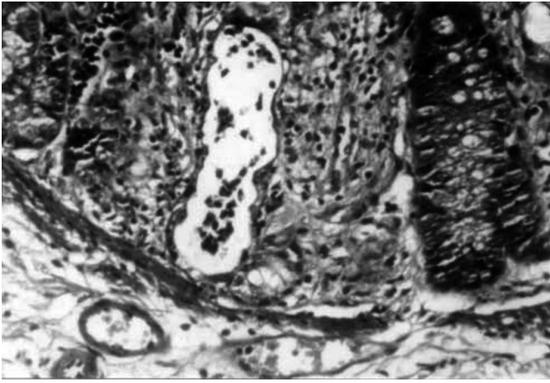


Figure 3 Crypt abscess and nuclear debris in a crypt lumen (HE X 310).

The pathological process, determined with necrosis and edema of the mucosa and focal mucosal ulcers continued till the 15th d in euthanized animals of control and formalin groups. Betamethasone group showed the lowest pathologic scores on the 15th d.

The mucosal cytokines IL-1 β , IL-2, IL-6 and IL-8 have been reported to play a significant role in the etiology of radiation-induced proctoproctitis^[30]. Mesalazine and betamethasone with cytokine suppressive effects have a curative outcome on this immunology-based disease, parallel to our results. The present study showed a solely descriptive basis of the histopathology, which determines the need and extent of the clinical therapy. The immunological contribution of the disease and the effect of therapeutic approaches need to be clarified.

It should be noted that two specimens were excluded from the study, one formalin group specimen on the 15th d due to lymphocytic colitis, one misoprostol group specimen on the 15th d due to lymphoid hyperplasia and submucosal edema. Since lymphocytic colitis, lymphoid hyperplasia and submucosal edema were encountered over the whole rectal wall of the two specimens from the same animal, the scoring of them was thus impossible. Mistol has radiation protective and tissue regenerating effects, but its mechanism of action is not fully understood^[8]. We showed that it could contribute to the healing process after irradiation.

Rectal formalin therapy has serious side effects like worsening of colitis, rectal pain, anal stenosis, rectal ulcers and anal incontinence^[4]. These side effects have been reported even in series with good clinical results^[5,14]. These effects are in accordance with our histopathologic findings.

In conclusion, formalin should not be used in order to avoid its toxic effects on mucosa. Mesalazine and betamethasone can be used for local therapy with no major superiority between each other. Controlled randomized prospective clinical trials are required to determine the best management of this disease.

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RAPID COMMUNICATION

Seroepidemiology of hepatitis C and its risk factors in Khuzestan Province, South-West of Iran: A case-control study

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extramarital sexual activities (2.4%), tattooing (3.6%) were found to be independent risk factors of being HCV-positive. No apparent risk factors could be demonstrated in 29 (11.2%) of the positive cases.

CONCLUSION: Our data indicate that a history of transfusion and iv drug abuse and haemodialysis are important risk factors for HCV infection in our area and that more careful pretransfusion screening of blood for anti-HCV must be introduced in our blood banks. Improvements in certain lifestyle patterns, and customs in this area may be essential to prevent transmission of the infection.

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Key words: Epidemiological patterns; Hepatitis C virus; Risk factors; South-West of Iran

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Abstract

AIM: To evaluate possible risk factors for the spread of hepatitis C infection and to analyze the characteristics of the epidemiological and clinical patterns among the patients with hepatitis C infection.

METHODS: During a five-year period a cross-sectional study was conducted among HCV positive individuals referred to the Ahwaz Jundishapur University Hospitals (AJSUH) and Hepatitis Clinic from 1 Sept 1999 to 1 Sept 2003. The control group consisted of first time blood donors referred to the Regional Blood Transfusion organization. Enzyme-linked immunosorbent assay and recombinant immunoblot assay anti-hepatitis C virus (HCV) tests were performed for two groups. Positive serum specimens were retested using polymerase chain reaction (PCR) for HCV RNA. Risk factors were evaluated using a questionnaire. Reported risk factors among infected subjects ("HCV-positive") were compared to those of subjects never exposed ("HCV-negative") to HCV.

RESULTS: A total of 514 subjects were studied for HCV, of which 254 were HCV-positive and 260 HCV-negative donors comprised the control group. Mean age of the patients was 28.4 (Std 15.22) years. HCV-positive subjects were more likely to be of male gender (63% versus 37%). Transfusion 132 (52%), non-intravenous (n-iv) drug abuse and iv drug abuse 37 (14.5%), haemodialysis 25 (10%), receiving wounds at war and

INTRODUCTION

Hepatitis C virus (HCV) infection is responsible for considerable morbidity and mortality worldwide. HCV is a leading cause of liver failure and liver transplantation in adults. Identified risk factors for HCV infection include intravenous (IV) drug use, exposure to infected blood products, and intranasal drug use^[1]. High-risk sexual activity [multiple sexual partners, history of sexually transmitted disease (STD)], tattooing, and skin piercing have also been suggested to be associated with increased risk for HCV^[2]. In addition, mother-to-infant transmission has been demonstrated^[3,4], but the possibility of other transmission routes has not been thoroughly explored. With the use of RT-PCR or bDNA techniques, HCV RNA has been detected in many systemic fluids other than blood, including peritoneal fluids, seminal and vaginal secretion, urine, and feces. These observations, however, have not been confirmed by all investigators^[5]. The possibility of HCV replication in the mosquito alimentary tract has recently been demonstrated, but the epidemiological importance of this has not yet been

determined^[6]. The rate of HCV infection differs in particular countries. The prevalence in developed countries amounts to 0.2%-2.2%, while in developing countries it reaches 7%. In some regions or risk group the rate of occurrence may be as high as 30%-90%. In Iran, according to the latest data, it is estimated that between 0.12-0.89 percent of the general population present anti-hepatitis C virus antibodies, which corresponds to as many as 0.5 million chronic carriers^[7,8].

Risk factors associated with HCV infection may be specific to a country or region^[9]. Few data are available about the risk factors associated with HCV infection in our area. Hepatitis C is a common health problem in South-West of Iran, and needs proper attention to alleviate the suffering of the people. It is essential to assess the magnitude of the problem, which will help us in understanding the dynamics of its transmission and can be utilized to guide screening procedures as well as provide insight into the control and prevention of the disease.

Herein, we report results of our cross-sectional analyses of risk factors for HCV infection in our community in Khuzestan Province, South-West of Iran conducted during a five year period.

MATERIALS AND METHODS

During a five-year period a cross-sectional study was conducted among HCV positive individuals referred to the Ahwaz JundiShapour University Hospitals (AJSUH) and Hepatitis Clinic from 1 Sept 1999 to 1 Sept 2003. On the basis of a specially designed protocol, standard commercially available tests and physical examinations were performed. The analysis included data of medical history, physical examination and periodic evaluation clinically and serologically.

All subjects were evaluated using a face-to-face questionnaire about demographic (gender and age) and socioeconomic (education) aspects, parenteral exposure to blood or blood products, social and sexual behavior, occupational exposure, intravenous drug use, tattooing, acupuncture, surgery, previous hospitalization and parenteral administration of drugs, personal history of jaundice or hepatitis or history of these diseases in the cases' and controls' families. The control group consisted of first time blood donors referred to the Regional Blood Transfusion Organization. None of the control group subjects were HBsAg positive, HIV-positive or have any signs or symptoms of hepatitis.

Enzyme-linked immunosorbent assay (Organon/Teknica UB/HCV EIA) and recombinant immunoblot assay anti-hepatitis C virus tests were performed for two groups. Positive serum specimens were retested using polymerase chain reaction (PCR) for HCV RNA.

None of our patients showed any signs of HBV infection, or any other cause of acute or chronic liver disease, such as HAV, EBV, CMV infections, auto-immune diseases, alcohol and drug abuse, α 1-antitrypsin deficiency, Wilson's disease, or hemochromatosis. HBsAg, anti-HBc were determined by IMx analyzer (Abbott Lab., Abbott Park, IL, USA). HBsAg, anti-HBc were determined by IMx analyzer (Abbott Lab., Abbott Park, IL, USA).

Table 1 Demographic features and symptoms and signs of liver disease of the patients enrolled into the study

Variable	HCV positive (<i>n</i> = 254)	
	<i>n</i>	%
Sex		
Male	160	63
Female	94	37
Age group (yr)		
< 20	81	32
20-29	82	32.5
30-39	56	22
40-59	26	10
> 60	9	3.5
Total	254	100
Symptoms		
RUQ Pain	142	56
Malaise	93	36.6
Dyspepsia	58	22.8
Signs		
Splenomegaly	25	9.8
Jaundice	12	4.7
Hepatomegaly	8	3.1
Elevated serum ALT level	168	66
HIV positive	3	1.2

Reported risk factors among infected subjects ("HCV-positive") were compared to those of subjects never exposed ("HCV-negative") to HCV. Consent for an interview was taken from each participant, who was assured about the confidentiality of his information. Controls were briefed about the known modes of HCV transmission. The institutional Ethics Review Committee approved the study protocol.

Statistical analysis

Collected data were coded, analyzed and computed, using the Statistical Package for Social Sciences (SPSS) version 10 (SPSS Inc., Chicago, IL, USA). Simple statistics such as frequency, and standard deviation were used. We conducted a multivariate logistic regression analysis to identify factors associated with HCV infection. Chi-square and Student's *t*-tests were used for comparison.

RESULTS

A total of 514 subjects were studied for HCV, of which 254 were HCV-positive and 260 HCV-negative donors comprised the control group. Mean age of the patients was 28.4 (SD 15.22) years. Of the 254 patients, 225 (88.6%) had identifiable risks of exposure to HCV infection.

HCV-positive subjects were more likely to be of male gender (63% *vs* 37%). Transfusion 132 (52%), non-intravenous (n-iv) drug abuse and iv drug abuse 37 (14.5%), haemodialysis 25 (10%), receiving wounds at war and extramarital sexual activities 6 (2.4%), tattooing (3.6%) were found to be independent risk factors of being HCV-positive. The mean age was significantly younger in patients with transfusion (13.4 years) than the mean age of all the patients (28.4 years, *P* = 0.004), mainly those with thalassemia and received regular blood transfusion. No

Table 2 Risk factors possibly associated with HCV-positive in patients group

Variable	HCV-negative (n = 260)		HCV-positive (n = 254)	
	n	%	n	%
Risk factor				
Blood transfusions	6	2.3	132	52
Intravenous drug use	0	0	37	14.5
Extramartial sexual activities	1	0.4	3	1.2
Tattooing	2	0.8	9	3.5
Surgical procedures	5	1.9	5	2
Dental procedures	8	3.1	12	4
Hemodialysis	0	0	25	10
Receiving wounds at war	0	0	3	1.2
Endoscopy	14	5.4	1	0.4
Unknown	224	86.1	29	11.2
Total	260	100	254	100

apparent risk factors could be demonstrated in 29 (11.2%) of the positive cases.

The demographic features and the background characteristics of the patient population are displayed in Table 1. Differences in the distribution of risk factors were compared between the HCV positive and negative groups, with similarities observed for educational level, economic status, and residency.

Transfusion ($P < 0.002$), intravenous drug use ($P < 0.005$) and hemodialysis ($P < 0.008$) were the only three variables with a statistically significant correlation with the presence of HCV infection on univariate analysis. There were no other statistically significant differences between the HCV positive and HCV negative control group in terms of demographic characteristics or topic areas associated with HCV infection/transmission such as extramarital sexual activities, tattooing, endoscopy, surgical and dental procedures (Table 2).

DISCUSSION

Chronic HCV infection represents one of the major public health problems in Iran and according to the annual IBTO internal reports, it is estimated to be less than 0.2%^[8,10]. Approximately 20%-30% of patients with chronic HCV infection will progress to cirrhosis^[11], which can be further complicated by hepatic decompensation and development of hepatocellular carcinoma(HCC)^[12,13].

In this study, 254 anti-HCV-positive patients between 1999 and 2003 were investigated. The ages of these patients, at the time of data analysis, ranged from 7 to 68 years with a mean age of 28.4 years. Our study included 160 males and 94 females, with a male:female ratio of 1:7. This male preponderance in HCV-infected patients was also reported by others^[14,15].

Our study indicated blood transfusion was the leading risk factor for HCV acquisition in our patients as 52% of them were diagnosed with chronic haemolytic anemias, mainly thalassemia and received regular blood transfusion. It should be emphasized that these cases most probably,

had contracted the infection before testing for HCV antibodies was performed routinely. Screening for HCV has been a routine for all blood donors since 1995 in Iran^[8]. Although it is not clear whether all the HCV-positive subjects in our study population with a positive history of transfusion had had their transfusion before 1995, the date of transfusion was not determined in our study. Therefore, more careful pretransfusion screening of blood for anti-HCV must be introduced in our blood banks.

Injection drug users (IDUs) constitute the largest group of persons at high risk for acquiring HCV infection in developed countries^[14,15]. Intravenous drug use was the second most frequent risk factor for HCV acquisition in our patient group (14.5%) and all of them were male, which is consistent with other reports. The male patients tended to have a history of IDU, whereas, female patients tended to have a history of transfusion^[16,17]. The data on route of drug administration were based on selfreporting. One must always consider the possibility that injection drug use is underreported and that some of the HCV infection among the non-injection drug users (NIDUs) may have actually occurred through injection of drugs^[18,19]. Although it is certainly possible that some of the NIDUs in this study may have injected drugs and become infected with HCV *via* injection of drugs, it does not appear that underreporting of injection drug use is a viable explanation for the results of this study.

As reported in several studies, HCV infection is a significant health problem in dialysis units in our country with a high prevalence rate (13.2%) among this population^[20]. It was reported that blood transfusion and duration of dialysis treatment are important risk factors for HCV infection in patients on haemodialysis^[21,22]. The more units transfused, the higher the risk for HCV infection.

Besides, we cannot exclude the possibility of nosocomial transmission of the virus over time in this group. This is supported by the fact that 8.8% of infected patients in this population had no history of blood transfusion. Patient-to-patient transmission was prospectively shown in some incidence studies in hemodialysis patients^[17]. Some strategies to reduce the risk of HCV infection in patients on hemodialysis such as early screening of patients for anti-HCV, reduction of transfusion by the use of erythropoietin or screening with more sensitive methods to detect HCV; and reducing the duration of the hemodialysis period by early transplantation should be considered in this group.

No apparent risk factors could be demonstrated in 29 (11.2%) of the positive cases (called sporadic infections), which is similar to those reported previously^[17]. In sporadic infections with no apparent risk factors, other routes of transmission and other factors, such as use of the same razor or tooth brush, or careless dressing of cuts and wounds or the family environment, where the infection risk increases along with exposure time should be noted^[24]. Another possible explanation for this percentage of sporadic infections is failure of the questioning process in our study. We found no significant relationship between sex, previous endoscopy, receiving wounds at war, marital status with the risk of HCV infection as independent risk

factors in our cases in comparison with controls.

Although the serological, epidemiological and possible risk factors of HCV infection here obtained were related to a small population, this study was justified by the lack of information about HCV infection in Khuzestan Province, South-West of Iran. Other limitations of the study are linked to the difficulties inherent in self-reporting of behaviors such as sexual activity and drug use. It is also possible that the patients receiving blood transfusion in the study may not be representative of the population of Khuzestan Province. Genotyping was not performed in our studied cases due to limitations in our area but the genotypes of HCV were investigated in Iranian patients with histologically proven chronic hepatitis, genotype (1a) has been identified in the majority of the patients^[25].

In conclusion, we provide epidemiologic features of hepatitis C and its risk factors in Khuzestan Province in South-West of Iran. These data are useful for understanding the risks of transfusion, hemodialysis, and other lifestyle patterns that predispose people to a number of HCV risk factors. These findings could be utilized to primary prevention program focused on identified risk factors, may help curtail the spread of HCV infection in this and other similar settings in developing countries. These results suggest that further study on the mode of transmission of hepatitis C should focus on patients who deny intravenous drug use or apparent risk factors. This information contributes to our understanding of the worldwide prevalence of hepatitis C. It demonstrates the importance to continue this study in order to establish routine procedures that can be applied to the screening and confirmation of the diagnosis, as well as to provide an applied methodology for the epidemiological investigation of the virologic profile of HCV-infected patients.

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RAPID COMMUNICATION

Triple, standard quadruple and ampicillin-sulbactam-based quadruple therapies for *H pylori* eradication: A comparative three-armed randomized clinical trial

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Abstract

AIM: To compare the effectiveness of triple, standard quadruple and ampicillin-sulbactam-based quadruple therapies for *H pylori* eradication in a comparative three-armed randomized clinical trial.

METHODS: A total of 360 *H pylori*-positive patients suffering from dyspepsia and aging 24-79 years with a median age of 42 years were enrolled in the study and randomly allocated into the following three groups: group A ($n = 120$) received a standard 1-wk triple therapy (20 mg omeprazole b.i.d., 1000 mg amoxicillin b.i.d., 500 mg clarithromycin b.i.d.); group B ($n = 120$) received a 10-d standard quadruple therapy (20 mg omeprazole b.i.d., 1000 mg amoxicillin b.i.d., 240 mg colloidal bismuth subcitrate b.i.d., and 500 mg metronidazole b.i.d.); group C ($n = 120$) received the new protocol, i.e. 375 mg sultamicillin (225 mg ampicillin plus 150 mg sulbactam) b.i.d. (before breakfast and dinner), instead of amoxicillin in the standard quadruple therapy for the same duration. Chi-square test with the consideration of $P < 0.05$ as significant was used to compare the eradication rates by intention-to-treat and per-protocol analyses in the three groups.

RESULTS: The per-protocol eradication rate was 91.81% (101 patients from a total of 110) in group A, 85.84% (97 patients from a total of 113) in group B, and 92.85% (104 patients from a total of 112) in group C. The intention-to-treat eradication rate was 84.17% in group A, 80.83% in group B, and 86.67% in group C. The new protocol yielded the highest eradication rates by both per-protocol and intention-to-treat analyses followed by the standard triple and quadruple regimens, respectively. However, the differences were not statistically significant between

the three groups.

CONCLUSION: The results of this study provide further support for the equivalence of triple and quadruple therapies in terms of effectiveness, compliance and side-effect profile when administered as first-line treatment for *H pylori* infection. Moreover, the new protocol using ampicillin-sulbactam instead of amoxicillin in the quadruple regimen is a suitable first-line alternative to be used in regions with amoxicillin-resistant *H pylori* strains.

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Key words: Triple therapy; Quadruple therapy; Ampicillin-sulbactam; *H pylori*

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INTRODUCTION

H pylori is responsible for the majority of peptic ulcer diseases and its eradication leads to the cure of such diseases, thereby eliminating the need for surgical treatment. Eradication of *H pylori* is indicated in the management of dyspepsia in patients under the age of 45 years without alarm symptoms (the 'test and treat' strategy) and also serves as a preventive treatment in precursor lesions of gastric cancer.

According to the Maastricht 2 guidelines, first-line eradication is triple therapy with the use of a proton-pump inhibitor b.i.d., 1 g amoxicillin b.i.d., and 500 mg clarithromycin b.i.d. In the case of penicillin allergy, 500 mg metronidazole b.i.d. is substituted for amoxicillin. When first-line *H pylori* eradication fails, a second-line treatment of quadruple therapy, with a proton-pump inhibitor b.i.d., colloidal bismuth subcitrate q.i.d., 500 mg metronidazole t.i.d., and 500 mg tetracycline q.i.d. is recommended.

Antibiotic resistance is the main cause of failure for *H pylori* eradication and beta-lactamase produced

by resistant *H pylori* strains is a possible mechanism underlying the ineffectiveness of an amoxicillin-based triple or quadruple therapy^[1]. Of the 153 clinical isolates of *H pylori* found in a previous study, 71.9% are resistant to amoxicillin, 77.8% to metronidazole, and 39.2% to both^[2]. The resistance rate to clarithromycin is currently 2%-30%^[3]. Consequently, new treatment modalities have recently emerged to overcome antibiotic resistance^[4]. However, comprehensive comparisons of the effectiveness of traditional and new treatment modalities are lacking in the literature.

Antibacterial activities of beta-lactamase inhibitors such as clavulanic acid and sulbactam have been demonstrated in a number of *in vitro* studies^[5,6]. However, using clavulanic acid associated with amoxicillin has not significantly increased the *H pylori* eradication rate *in vivo*^[7,8]. The aim of this study was to compare the effectiveness of the following therapeutic regimens (triple therapy, standard amoxicillin-based quadruple therapy, ampicillin-sulbactam-based quadruple therapy) in eradicating *H pylori* in a three-armed randomized clinical trial for the first time.

MATERIALS AND METHODS

Patients and medications

A total of 360 *H pylori*-positive patients suffering from dyspepsia and aging 24-79 years with a median age of 42 years were enrolled in the study. *H pylori* status was determined by rapid urease test at entry. After giving written informed consent, the patients were randomly allocated into three groups: group A ($n = 120$) received a standard 1-wk triple therapy (20 mg omeprazole b.i.d., 1000 mg amoxicillin b.i.d., 500 mg clarithromycin b.i.d.); group B ($n = 120$) received a 10-d standard quadruple therapy (20 mg omeprazole b.i.d., 1000 mg amoxicillin b.i.d., 240 mg colloidal bismuth subcitrate b.i.d., and 500 mg metronidazole b.i.d.); group C ($n = 120$) received 375 mg sultamicillin (225 mg ampicillin plus 150 mg sulbactam, purchased from Pfizer SA, Case Postale, 8048 Zurich, Switzerland) b.i.d. (before breakfast and dinner), instead of amoxicillin in the standard quadruple therapy for the same duration. *H pylori* eradication was confirmed by C¹⁴-urea breath test following 6 wk from the end of therapy.

All patients were contacted periodically, asked about the occurrence of possible side effects, and appropriate guidance was provided when needed. Those who were lost to follow up, or used antibiotics in the time period between the end of therapy and post-treatment urea breath test, or could not complete the treatment course because of severe side effects, were excluded in the per-protocol analysis.

Statistical analysis

The study was a three-armed randomized clinical trial with groups A, B and C including 110, 113 and 112 patients for the final statistical analysis (per-protocol analysis). In addition, intention-to-treat analysis was also performed. Chi-square test was used to compare the eradication rates by intention-to-treat as well as per-protocol analyses in the three groups. $P < 0.05$ was considered statistically significant.

Table 1 Demographic and clinical data of patients who completed the treatment

	Group A (Triple therapy)	Group B (Quadruple therapy)	Group C (New protocol)
Patients (<i>n</i>)	110	113	112
Male, <i>n</i> (%)	57 (52)	60 (53)	62 (55)
Female, <i>n</i> (%)	53 (47)	53 (47)	50 (45)
Age range (yr)	28-79 (median 41)	24-47 (median 39)	31-68 (median 47)
Minor side effects, <i>n</i> (%)	6 (5)	5 (4)	12 (11)
Overall eradication (<i>n</i>)	101	97	104
Intention-to-treat (%)	84.17	80.83	86.67
Per-protocol (%)	91.81	85.84	92.85

RESULTS

Five patients in group A, 3 in group B, and 4 in group C were lost to follow up. Four patients in group A, 2 in group B, and 4 in group C used antibiotics in the time period between the end of therapy and post-treatment urea breath test. One patient in group A and 2 in group B discontinued the regimen due to severe allergic reactions. Minor side effects were experienced by 6 patients in group A (vomiting, skin rash and abdominal pain), 5 patients in group B (vomiting, skin rash and pruritis) and 12 patients in group C (vomiting, diarrhea, headache, skin rash and abdominal pain).

Demographic and clinical details of the patients remaining in the three groups are shown in Table 1. The per-protocol eradication rate was 91.81% (101 patients from a total of 110) in group A, 85.84% (97 patients from a total of 113) in group B, and 92.85% (104 patients from a total of 112) in group C. The intention-to-treat eradication rate was 84.17% in group A, 80.83% in group B, and 86.67% in group C. The new protocol yielded the highest eradication rates by both per-protocol and intention-to-treat analyses followed by the standard triple and quadruple regimens, respectively. However, the differences were not statistically significant between the three groups. They were also not significantly different in the occurrence of minor side effects, either.

DISCUSSION

We opted to prescribe antibiotics for ten days because 4- and 7-d regimens have been unsuccessful in Iran^[9]. In this study, the eradication rates for the triple, standard quadruple and ampicillin-sulbactam-based quadruple therapies were not significantly different. Occurrence of serious side effects necessitating termination of therapy was negligible in all three groups. Minor side effects were well tolerated among all three groups and occurred infrequently with almost the same frequency. Diarrhea and headache occurred in group C only, but other side effects were experienced in all groups.

Some recent studies have compared the efficacy of triple versus quadruple therapy, and a recent meta-analysis

has assessed these studies^[10]. Eradication rates were not significantly different among patients receiving triple or quadruple therapy. The duration of therapy (7 vs 10 d) did not significantly change the results, either. Triple therapy given for a 10-d period achieved an intention-to-treat eradication rate of 79% compared with 77% for a 7-d period. Quadruple therapy on the other hand gave an intention-to-treat eradication rate of 83% for a 10-d period and 80% for a 7-d period^[10]. The eradication rates by intention-to-treat analysis among patients receiving either triple or quadruple therapy in this study were almost similar to those obtained previously^[4,10,11].

A previous preliminary study by the authors using ampicillin-sulbactam instead of amoxicillin in 10-d standard quadruple therapy on 26 patients has yielded a 92% eradication rate by per-protocol analysis which was well tolerated among patients (unpublished data). The present study is the first randomized clinical trial to evaluate the efficacy of the new protocol and to compare it with standard triple and quadruple therapies in a relatively large number of patients. Although not statistically significant, the new protocol seems to be more effective than traditional protocols.

H pylori infection has a high prevalence rate of about 90% in Iran, which emphasizes the importance of having an effective regimen to eradicate *H pylori*^[12]. The metronidazole-based standard triple therapy regimen has been unsuccessful in *H pylori* eradication, yielding an eradication rate of only about 55% compared with about 90% in other countries^[13,14]. This is because metronidazole-resistant *H pylori* strains are rather common in Iran as well as in other developing countries^[9,15]. The high prevalence of metronidazole-resistance in Iran could be explained by the frequent use of metronidazole to treat various infections, thereby promoting antibiotic resistance in *H pylori*.

On the other hand, 7.4% of *H pylori* isolates in Iran have been reported to be resistant against amoxicillin and higher resistance rates of up to 29% have been reported in other developing countries^[15,16]. Therefore, the use of ampicillin-sulbactam instead of amoxicillin in the quadruple therapy regimen, leading to an eradication rate of 92.85% by per-protocol and 86.67% by intention-to-treat analysis in this study, may be useful against metronidazole- and amoxicillin-resistant *H pylori* strains in developing countries like Iran. Consequently, there would be no need to exclude metronidazole (because of antibiotic resistance), which is an inexpensive and widely available anti-*H pylori* agent in developing countries.

Since the present study did not show the effectiveness of the new combination on ampicillin-resistant strains, we should bear in mind that some of the resistant strains do not act through beta-lactamase but rather penicillin binding proteins (PBPs)^[17]. Perhaps *in vitro* study of ampicillin-resistant strains using ampicillin-sulbactam combination can help answer whether the combination is effective against the resistant strains.

In conclusion, the results of this study provide further support for the equivalence of triple and amoxicillin-based quadruple therapies in terms of effectiveness, compliance

and side-effect profile when administered as a first-line treatment for *H pylori* infection. Moreover, the new protocol using ampicillin-sulbactam instead of amoxicillin in the quadruple regimen is a suitable first-line alternative to be used in regions with amoxicillin-resistant *H pylori* strains.

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RAPID COMMUNICATION

Prolonged intestinal mucosal acidosis is associated with multiple organ failure in human acute pancreatitis: Gastric tonometry revisited

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Abstract

AIM: To evaluate whether multiple determinations of intramucosal pH (pHi) in acute pancreatitis (AP) patients could provide additional information of the disease severity during early hospitalization.

METHODS: Twenty-one patients suffering from acute pancreatitis were monitored by gastric tonometry in the first 72 h after hospital admission.

RESULTS: In the survivor group ($n = 15$) the initially low pHi values returned to normal level ($\text{pHi} \geq 7.32$) within 48 h (median pHi: d 1: 7.21; d 2: 7.32; d 3: 7.33). In contrast, pHi values in the non-survivor group ($n = 6$) were persistently either below or in the low normal range (median pHi 7.12; 7.12; 7.07 respectively), but pHi differences between the two groups reached significance only after 24 h ($P < 0.01$). Mucosal acidosis detected at any time during the monitored period was associated with the emergence of single or multiple organ dysfunction ($P < 0.01$).

CONCLUSION: Prolonged gastric mucosal acidosis was associated with remote organ dysfunction and failure in Acute Pancreatitis, however, correlation with the fatal outcome became significant only 24 h after admission. Due to its non-invasive nature gastric tonometry may supplement the pro-inflammatory markers to achieve a multi-faceted monitoring of the disease.

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INTRODUCTION

Necrotizing acute pancreatitis (AP) is currently recognized as a two-phase systemic disease, where the early phase (within the first two weeks) is characterized by sterile pancreatic necrosis and concomitant development of systemic inflammatory response syndrome (SIRS). If the patient survives the early MODS, the disease may progress to a second phase with the development of infected pancreatic necrosis and septic complications associated with multiple organ failure (MOF)^[1].

Since an abundance of Gram negative bacteria^[2] and other pathogens of gastrointestinal origin are commonly detected in pancreatic infections, the gut is considered to be the main source of pancreatitis related septic complications^[3]. This is in accordance with experimental data suggesting that bacterial translocation through the damaged gut barrier is the most important mechanism for the contamination of pancreatic necrosis^[4]. Recent evidence indicates that in case of host stress and low availability of intestinal luminal nutrition, non-pathogenic commensal intestinal bacteria can rapidly switch on virulence genes and mount a toxic offensive for their survival. Therefore, when highly virulent traits of enteric bacteria emerge by virulence phenotype switching, gut-derived sepsis may be more likely to occur in severe AP^[5].

Severe AP associated intestinal mucosal dysfunction may be the consequence of several deleterious local and systemic factors, such as disturbance of perfusion (ischemia-reperfusion phenomenon), oxidative stress during systemic inflammatory response syndrome, absence of mucosal feeding during parenteral nutrition, etc. This will eventually lead to metabolic failure in the gut, inducing intramucosal acidosis. Measurement of gastric intraluminal pCO₂ by

balloon tonometry and calculation of intramucosal pH (pHi) provides a quantitative indicator of the adequacy of intestinal milieu. Several studies have confirmed the value of pHi as a predictor of morbidity and mortality in the critically ill^[6].

Gastric tonometry has been applied in human acute pancreatitis, however, the available clinical data are limited. The aim of this study was to analyze the relationship between intramucosal acidosis and AP associated complications (remote organ dysfunction and infection) with special attention to the outcome of the disease.

MATERIALS AND METHODS

Patients

The Ethics Committee of Semmelweis University Medical School has approved the study protocol, and written informed consent was obtained from each patient.

Gastric tonometry is generally considered to be useful in critically ill patients therefore the enrollment into this study was focused on patients hospitalized for suspected severe acute necrotizing pancreatitis. For control purposes patients with moderate and mild severity of pancreatitis were also included. The diagnosis of AP was based on laboratory findings and imaging studies (abdominal ultrasound and/or computer tomography) in association with the typical clinical picture. Exclusion criteria were as follows: pancreatitis associated with pancreatic cancer, history of recurrent AP within 3 mo, chronic, post-traumatic or post-operative pancreatitis, childhood, pregnancy, as well as administration of immune suppressive drugs (including steroids) in the previous one month. Twenty-one patients suffering from acute pancreatitis were enrolled; they were either admitted to the surgical intensive care unit (ICU) or to general surgical ward, all of them were monitored by gastric tonometry for the first three days. The end point of the study was the outcome of the disease. Based on this two groups were created in a retrospective fashion; patients with fatal outcome were enrolled in Group 1, whereas patients who survived were enrolled in Group 2.

All patients were treated according to the usual AP protocols adjusted to their current condition. Initial management was conservative. During the acute phase, the therapy consisted of adequate fluid replacement through a central venous catheter with hemodynamic monitoring, and assistance of respiratory or renal function if needed. A modified nasogastric tube was inserted to keep the stomach empty and measure intraluminal pCO₂. Analgesics were given as required to all patients, and they received proton pump inhibitor (PPI) (2 × 40 mg omeprazole intravenously) to prevent stress ulcers. Prophylactic antibiotics (ofloxacin/metronidazole or imipenem) were administered as soon as the presence of necrosis was evident, or CRP value was over 150 mg/L. Surgical intervention was performed when infected necrosis was diagnosed (2 cases), or if the patient's condition deteriorated despite intensive medical therapy (1 case). In one case laparoscopic cholecystectomy was performed after the patient's recovery from AP.

All patients were monitored by gastric tonometry at least twice daily during the first three days of hospitalization. Attending clinicians did not use the pHi values to guide the patients' management.

Gastric Tonometry

Gastric tonometry was performed using a semi-automated method^[7] according to the modification of the original technique described by Fiddian-Green *et al.*^[8]. A tonometry tube (Tonometrics™ 16F Catheter, Datex-Ohmeda Division, Instrumentarium Corp., Helsinki, Finland) was inserted into the lumen of the stomach via the nasogastric route. This catheter is a nasogastric tube with an additional smaller diameter conduit equipped with a CO₂ permeable silicone balloon attached to the tip. The balloon was inflated with room air and its line was connected to a bedside CO₂ monitor (Tonocap™ Monitor, Datex-Ohmeda). Gastric intraluminal pCO₂ pressure was measured after an equilibration period, and actual pHi was automatically calculated using additional data (arterial pH, arterial pCO₂) obtained from the patient's arterial blood samples. Since it is generally presumed that systemic metabolic acidosis confounds the interpretation of gastric pHi, all the pHi values were excluded from analysis where systemic metabolic acidosis was present. We have applied the simplified gastric pHi measurement protocol proposed by Bonham *et al.*, where the daily lowest pHi value was used to characterize the mucosal condition^[9]. Routine PPI administration has been employed to improve the accuracy of mucosal pCO₂ measurements^[9], since gastric acid secretion blockade prevents the spurious elevation of luminal pCO₂ (resulting from the artifactual carbon-dioxide accumulation due to the reaction of bicarbonate with acid derived H⁺-s). Mucosal acidosis was diagnosed if pHi ≤ 7.32 (the generally accepted cut off value^[11]).

Laboratory investigations

Serum amylase and lipase activities were measured by the standard spectrophotometry method (reagents from Boehringer Diagnostics, Mannheim, Germany), where the upper limit of normal values was 220 U/L and 190 U/L respectively. Acute pancreatitis was diagnosed if serum amylase level exceeded 600 U/l. CRP was determined routinely by immunoturbidimetric assay (Orion Diagnostica, Espoo, Finland); the upper limit of normal value was 10 mg/L.

Statistical analysis

All data were presented as: median (range). Statistical analysis was performed using the Mann-Whitney *U*-test. Results were considered significant if *P* values were < 0.05.

RESULTS

Twenty-one patients with acute pancreatitis (6 women and 15 men) were involved in the study with a median age of 60 (29-77) years. The overall mortality rate was 6 of 21. There were no significant differences between the non-survivor (Group 1) and survivor (Group 2) groups in median age, gender and duration of symptoms before hospital admission. Ranson's and modified Glasgow scores showed no significant differences between the two groups, in contrast, APACHE II scores were significantly higher in the non-survivor group from the second day after admission. Ethanol was the leading etiological factor in the first group, whereas biliary origin in the second (Table 1).

In the non-survivor group 3 of the 6 patients died

Table 1 Clinical characteristics of the patients investigated in this study

	Group 1 non-survivors (n = 6)	Group 2 survivors (n = 15)	P value
Age (yr) ¹	69 (41-77)	52 (29-69)	0.22
Sex ratio (M/F)	5/1	10/5	
Duration of symptoms before admission (h) ¹	49.5 (12-104)	17 (4-120)	0.09
Etiology			
Ethanol/gallstone/other	3/2/1	5/8/2	
Disease severity			
Predicted severity by Ranson's score ¹	5.5 (1-8)	3 (2-7)	0.18
Predicted severity by Glasgow criteria ¹	2.5 (2-6)	2 (0-6)	0.45
APACHE II at the time of admission ¹	12 (4-15)	6 (3-16)	0.128
APACHE II on the third hospital day ¹	14 (10-29)	7 (0-10)	0.005
Outcome			
Necrosis/No necrosis/No data ²	5/0/1	5/9/1	
Remote organ dysfunction ¹	3 (2-5)	0 (0-2)	0.0016

¹ Values are represented as median (range). APACHE: Acute Physiology and Chronic Health Evaluation. ² No CT scan or autopsy proven data of necrosis was available.

within the first 3 d (two patients due to cardiorespiratory failure, whereas the third one due to early MOF). Three other patients deceased later (on d 9, 17, 18 respectively), all of them developed sepsis and late MOF (Table 1.) Bacteria responsible for fatal septic complications were Klebsiella, Staphylococci, Enterococci, and unidentified Gram-positive cocci.

In the survivor group local complications (peripancreatic fluid collection, necrosis) evolved in 5 of 15 patients (Table 1), and remote organ dysfunction (circulatory, pulmonary) was observed in 6 cases. Blood culture positivity was observed in 8 of 15 patients. Klebsiella, Enterobacter, Pseudomonas aeruginosa, Staphylococci and alpha hemolytic Streptococci were identified in the samples.

At the time points when the tonometry measurements were performed, serum CRP levels were significantly higher in Group 1. Other inflammatory markers, such as white blood cell counts, and the manifestations of the systemic metabolic disturbances as reflected by the daily lowest arterial pH and actual base excess values did not differ significantly between the two groups (Table 2).

Using our pHi measurement protocol there were neither failures of tonometer placement, nor tonometer related complications.

In general, the daily lowest pHi values were permanently below 7.32 in the non-survivor group (Group 1), whereas pHi was normal, or only temporarily low in the survivor group (Group 2). The pHi value differences between the two groups did not reach statistical significance on the first hospital day (Figure 1); in contrast, on the second and third days pHi values in non-survivors became significantly lower (pH 7.12 and 7.07 respectively), than those of the survivors (Figure 1).

If the relationship between the daily lowest pHi values and the APACHE II scores of the corresponding day were

Table 2 Selected markers of inflammation, values of gastric tonometry measurements, blood test results in the patient groups

	Group 1 non-survivors (n = 6)	Group 2 survivors (n = 15)	P value
Markers of inflammation			
C-reactive protein (mg/L) ¹	408 (181-427)	234 (95-317)	0.048
White blood cell count (G/L) ¹	19.1 (14.5-22.6)	15.8 (10.2-23.2)	0.14
Positive bacterial hemoculture	3	8	
Gastric Tonometry			
Lowest pHi within the first 72 h ¹	7.11 (6.97-7.32)	7.31 (6.94-7.46)	0.0001
Lowest pHi during the third day ¹	7.07 (6.97-7.25)	7.33 (7.27-7.46)	0.0004
Arterial blood gas results			
Arterial pH ^{1,2}	7.35 (7.18-7.46)	7.38 (7.34-7.44)	0.18
Actual base excess (mmol/L) ^{1,2}	-9.4 (-12.5 - -4.5)	-7 (-13.3 - -1)	0.11

¹ Values are median (range). pHi: Calculated gastric intramucosal pH. ² Daily minimum values within the first 72 h.

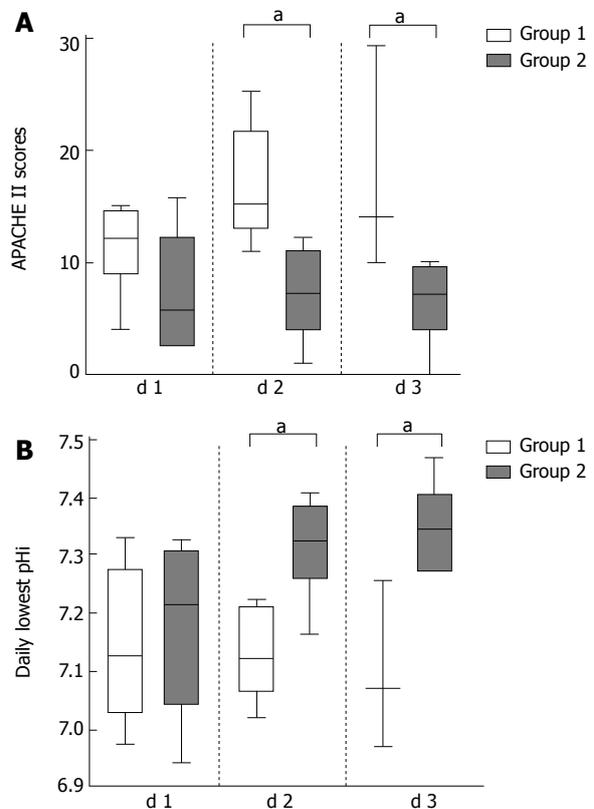


Figure 1 The daily lowest pHi and APACHE II values in acute pancreatitis in the survivor and non-survivor group in the first three days.

analyzed, the non-survivor group was characterized by a day-by-day decrease of pHi, which was associated with the expected rise in the APACHE II scores. Conversely, the originally subnormal pHi values of the survivor group have returned to the normal range with a corresponding decrease in the APACHE II scores (Figure 1).

We have found that assessment of the daily lowest pHi value was able to predict the development of non-infectious complications of acute pancreatitis. Prolonged mucosal acidosis -reflected by constantly subnormal pHi values- was associated with the development of MODS and fatal outcome. In contrast,

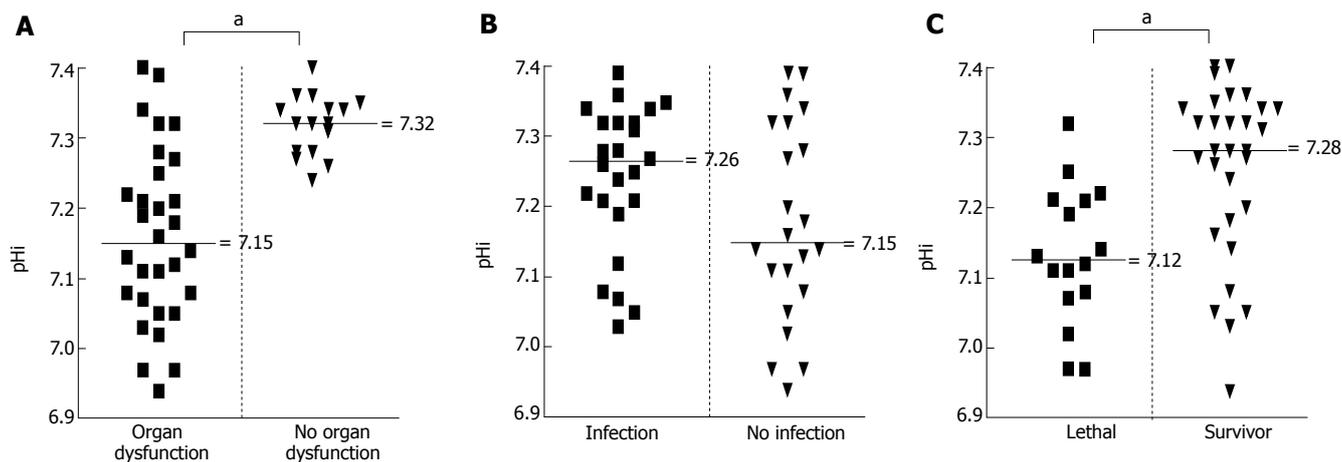


Figure 2 Evaluation of the predictor capacity of pHi values, if single measurements are considered.

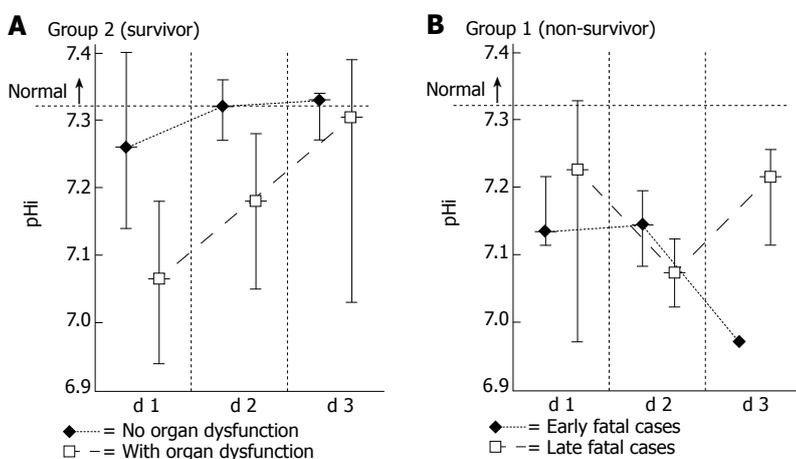


Figure 3 Dynamics of the daily lowest pHi values in relation to the different outcomes of acute pancreatitis. In the survivor group (Group 2) pHi data were sorted to reflect the emergence or absence of MODS. In the absence of MODS pHi values stayed close to the physiological range, returning from the initial depression to normal by d 3. In patients with MODS initial pHi values were considerably lower, but a return to normal values was detectable. In the non-survivor group (Group 1) each patient developed MOF, therefore values were sorted to reflect the early or late appearance of this complication. All pHi values continued to stay at subnormal levels from the day of admission, and never returned to normal. (Values represent median and range).

in this study low pHi values were unsuitable to predict infections (Figure 2).

We have also analyzed the dynamics of the daily lowest pHi values from the viewpoint of the development of multiple organ dysfunction/failure (Figure 3). The pHi data in the survivor group were subdivided to represent the emergence or absence of MODS. By definition, MODS in this group was responding to treatment, therefore it was transient in nature. The pHi data curve in the subgroup with the absence of remote organ dysfunction characteristically started slightly below the normal value and returned to normal level by d 3. In contrast, in the subgroup of patients with remote organ dysfunction the curve started low, but similarly, a return to normal values was noted.

Since in the non-survivor group (Group 1) each patient developed MODS with deterioration into MOF (not responding to treatment), the values were sorted to reflect the early or late appearance of this complication. Although such review of the data had limited statistical power (due to the low number of cases), it could be concluded that all pHi values -irrespective of the early or late emergence of MODS/MOF- stayed at subnormal levels from d 1 and never returned to normal.

DISCUSSION

In this study we have shown a strong correlation between

persistent low gastric mucosal pHi and therapy resistant MOF associated with severe AP. This finding is in accordance with the original results of Bonham *et al*⁹¹. They suggested that if the lowest pHi -measured at any time during the hospitalization- was below the 7.25 cut-off value, such severe mucosal acidosis was predictive of mortality in AP.

In a study by Juvonen *et al*¹²¹ pHi values generally did not discriminate mild AP from the severe form, but the pHi values measured at 48 h were significantly lower in the severe patient group. Our data showed that the course of gastric pHi alterations was indeed of considerable interest, since not only in mild cases, but also in patients with responsive severe disease, the originally low values returned to the normal range. This was in sharp contrast with the persistently subnormal pHi values characterizing the therapy resistant MOF cases. With respect to the early predictive capacity of gastric tonometry, however, pHi values at the time of admission did not prognosticate the outcome.

Recent advances in the comprehension of intestinal mucosal pathophysiology in AP deserve further comments. Previous studies used a somewhat simplistic framework of low-flow state and regional perfusion failure as being responsible for the development of mucosal acidosis and the related complications.

Bonham's group proposed that a mucosal ischemia-reperfusion injury was the culprit of gut barrier failure in

AP. Soong *et al*^[13] emphasized the role of hyperinflammation in the pathomechanism of mucosal injury, since peak endotoxin concentrations were detected before pHi fell to its lowest level. Mucosal acidosis correlated with the consumption of endotoxin core IgM antibodies as a reflection of antecedent circulating endotoxin exposure. Hynninen *et al*^[14] have found that intramucosal acidosis in severe AP was concomitant with, or rapidly followed by increases in circulating cytokines (IL-6, IL-8, and IL-10), but they could identify no correlation between endotoxemia and low pHi.

It can be suspected that further -yet unexplored- elements may influence the mucosal integrity in AP including nitric oxide dependent vasoregulatory imbalance^[15], mucosal oxidative stress, up-regulation of cellular adhesion molecules, and activation of adherent polymorphonuclear leukocytes with consecutive, destructive oxygen free radical production, as well as alteration of intestinal bacterial virulence^[16,17]. Consequently, acidic gastric pHi should not be considered as a simple sign of the splanchnic bed hypoperfusion, but rather as a more complex pathophysiological representation of the involvement of the gut in SIRS, evolving together with the stress related alteration in the intestinal milieu.

Although our concept of mucosal barrier failure and bacterial invasion has been enriched by new details recently, the information concerning the general well-being of the mucosa provided by gastric tonometry still seems to be of value in cases of severe AP. Cumulating evidence suggests, however, that on the other side of the mucosal barrier (i.e. in the blood and tissues) a relative immune-deficient state evolves independently from mucosal acidosis in the late course of the disease. The exaggerated SIRS is followed by compensatory anti-inflammatory response syndrome (CARS) leading to immune deactivation^[18], in which the activation induced cell death (AICD) of polymorphonuclear leukocytes^[19] may be an important constituent.

The additive effects of these key elements may well represent the decisive step towards sepsis, and indeed, gastric tonometry will reflect the mucosal part only. (Despite the apparent mucosal dysfunction detected in our severe AP cases, a statistically significant association between low pHi values and infectious complications could not be established in this study.)

In conclusion, intramucosal pH values showed characteristic, time dependent alterations distinguishing mild acute pancreatitis from the severe form, but detection of mucosal acidosis at admission did not improve outcome prediction. The complex sequence of pathophysiological events responsible for the development of intramucosal acidosis and septic complications is not fully elucidated in acute pancreatitis. However, future investigations should continue to benefit from gastric tonometry as a non-invasive adjunct in monitoring the intestinal mucosal function during the course of the disease.

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Influence of iron on the severity of hepatic fibrosis in patients with chronic hepatitis C

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Abstract

AIM: To evaluate the association among hepatic fibrosis, serum iron indices, and hepatic iron stores in patients with Chronic Hepatitis C (CHC).

METHODS: Thirty-two CHC patients were included in our study. The histological degree of fibrosis and inflammation activity was assessed according to the Metavir system. The serum iron indices including ferritin, iron and transferrin saturation were measured. Hepatic iron deposition was graded by Perls' stain.

RESULTS: The CHC patients with severe hepatic fibrosis ($n = 16$) were significantly older than CHC patients with mild fibrosis ($n = 16$) ($P = 0.024$). The serum iron indices, increased serum iron store and positive hepatic iron stain were not significantly different between the two groups. In multivariate logistic regression analysis, the age at biopsy was an independent predictor of severe hepatic fibrosis (Odds ratio = 1.312; $P = 0.035$). The positive hepatic iron stain was significantly associated with the values of alanine aminotransferase (ALT) ($P = 0.017$), ferritin ($P = 0.008$), serum iron ($P = 0.019$) and transferrin saturation ($P = 0.003$). The ferritin level showed significant correlation with the value of ALT ($r = 0.531$; $P = 0.003$), iron ($r = 0.467$; $P = 0.011$) and transferrin saturation ($r = 0.556$; $P = 0.002$).

CONCLUSION: Our findings suggest that the severity of hepatitis C virus (HCV)-related liver injury is associated with patient age at biopsy. Both serum iron indices and hepatic iron deposition show correlation with serum indices of chronic liver disease but are not related to

INTRODUCTION

Elevations in serum iron, ferritin and transferrin saturation are common in patients with chronic hepatitis C (CHC), as are mild increases in hepatic iron concentration. It has been reported that up to 40%-46% of patients has elevated serum iron, ferritin, or transferrin saturation level^[1,2]. Although the degree of iron deposition is usually mild, histological evidence of liver iron accumulation can be observed in 10%-42.1% of patients with CHC^[2-4]. Increased amounts of iron in the liver may promote the progression of liver disease by adding oxygen free radicals that increase oxidative stress^[5,6]. Iron overload is responsible for liver damage through the generation of reactive oxygen species leading to lipid peroxidation and alteration of the cellular membrane^[7]. Therefore, iron overload may play a role in the pathogenesis of some chronic liver diseases, especially when iron is combined with other hepatotoxic factors such as virus, free fatty acid, and alcohol^[8]. In addition to the production of oxidative stress, the iron may enhance the rates of viral replication and impair the host immune system^[6]. Despite these observations, the exact role of iron overload in patients with CHC remains unclear.

Factors that increase the risk of progression of hepatitis C virus (HCV)-associated hepatic fibrosis include older age at infection, male sex, alcohol abuse, and concurrent viral infection, particularly with human immunodeficiency virus or hepatitis B virus^[9]. The influence of viral load and genotype on the pathogenesis of liver disease is not completely resolved. Most studies have reported that HCV RNA level has no relation to the activity of liver disease^[10]. There are 6 major HCV genotypes. The most types are type 1a, 1b, 2a, 2b in Taiwan and about 65% of HCV

infections are type 1b^[11]. In early studies, HCV genotype 1b was found to be associated with a more severe liver disease^[12]. However, the association between genotype 1b and a more severe liver disease had not been found in studies with adjustment for the confounding factors^[10,13].

Whether the degree of hepatic iron deposition in patients with CHC affects the natural history of the disease remains to be determined. The aim of this study was to assess the association among hepatic fibrosis and serum iron indices, hepatic iron stores in patients with CHC. This study was also performed to assess the other potential factors related to the severity of hepatic fibrosis in these patients, including age, gender, liver enzyme tests, viral load and genotype of HCV. We had adjusted for the other confounding factors, such as alcohol abuse, obesity, and concurrent human immunodeficiency virus or hepatitis B virus infection.

MATERIALS AND METHODS

CHC patients

The patients with CHC were collected at our outpatient department since October 2003. CHC was diagnosed by alteration in liver enzymes persisting for more than 6 mo associated with positive HCV antibody. Patients with potentially secondary causes of iron overload were excluded, including alcohol abuse (ethanol consumption > 20 g/d), ribavirin therapy, and multiple transfusions. The body mass index (BMI) of the patients was not over 27 kg/m². Co-infection with human immunodeficiency virus or hepatitis B virus was also excluded. Serum levels of ceruloplasmin were within normal range. Serological tests for autoimmune hepatitis (anti-nuclear antibody, anti-smooth muscle antibody) and for primary biliary cirrhosis (anti-mitochondrial antibody) were negative.

Serological evaluation

The serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined by using an Olympus 5000 analyzer. The upper limit of normal for ALT is 34 IU/L. HCV antibody was detected by a commercially available enzyme-linked immunosorbent assay (AxSYM. ABBOTT Diagnostic Corporation, USA). The iron status of each patient was evaluated by biochemical tests. Serum iron (normal range, 60-160 µg/dL) was measured by the colorimetry and ferritin (normal range: 18-274 ng/mL in men and 6-283 ng/mL in women) was measured by a commercially available enzyme-linked immunosorbent assay (AxSYM. ABBOTT Diagnostic Corporation, USA). Transferrin saturation was calculated as (the serum iron divided by the TIBC) × 100%. The increased serum iron store was defined by transferrin saturation > 50% and/or ferritin > upper normal limit.

Hepatic fibrosis stage

The hepatic specimens were obtained with the SURECUT needle by ultrasonography-guided biopsy of liver. The degree of fibrosis and inflammation activity was assessed according to the Metavir system^[14]. The Metavir system scores both necroinflammatory changes on a 4-point scale

of 0 to 3 and fibrosis on a 5-point scale from 0 to 4.

Hepatic iron deposition

The histological assessment of hepatic iron stores was revealed by Perls' stain on liver biopsy specimens^[15]. Hepatic iron deposition was graded on a scale from 0 to 4. Perls' stain is also called Prussian Blue reaction. It is used to demonstrate ferric iron and ferritin. This is not a true staining technique rather, it is a histochemical reaction. The protein is split off by the hydrochloric acid, allowing the potassium ferrocyanide to combine with the ferric iron. This forms the ferric ferrocyanide or Prussian Blue.

Viral load of HCV

The viral load of HCV was checked according to the Cobas Amplicor HCV Monitor Test, version 2.0, Roche Diagnostics. It is based on five major processes: specimen preparation; reverse transcription of the target RNA to generate complementary DNA (cDNA); polymerase chain reaction (PCR) amplification of target cDNA using HCV specific complementary primers; hybridization of the amplified products to oligonucleotide probes specific to the targets; and detection of the probe-bound amplified products by colorimetric determination.

Viral genotype of HCV

The most viral genotypes of HCV are type 1a, 1b, 2a, 2b in Taiwan. We used the method of type-specific PCR to analyze the viral genotype of HCV. Based on variation in nucleotide sequence within restricted regions in the putative C (core) region of HCV, four groups of HCV had been illustrated^[16]. They were types 1a, 1b, 2a and 2b. The method depended on the amplification of a C gene sequence by PCR using a universal primer (sense) and a mixture of four type-specific primers (antisense). HCV types were determined by the size of the products specific to each of them. The primers of first round PCR were 5'-CGAAAGGCCTTGTGGTACTG-3' and 5'-ATATACCCCATGAGGTCGGC-3'. The primers of second round PCR were sense primer 104: 5'-AGGAAGACTTCCGAGCGGTC-3' and four antisense primers. They were antisense primer 132: 5'-TGCCTTGGGGATAGGCTGAC-3', antisense primer 133: 5'-GAGCCATCCTGCCACCCCA-3', antisense primer 134: 5'-CCAAGAGGGACGGGAACCTC-3' and antisense primer 135: 5'-ACCCTCGTTTCCGTACAGAG-3'.

Statistical analysis

Data were summarized as mean ± SD. Data were compared between groups on the basis of hepatic fibrosis stage. Categorical variables were compared with the chi-square test or Fisher's exact test as required. Continuous variables were compared between groups by using the unpaired *t*-test. The Mann-Whitney test was used when it was appropriate. Independent factors related to hepatic fibrosis severity were assessed by using multivariate logistic regression analysis. Correlations among selected variables were assessed by the Spearman correlation coefficient. The *P* < 0.05 was statistically significant.

Table 1 Demographic and laboratory data of 32 patients with CHC

Variable	Total population (n = 32)	Severe fibrosis (n = 16)	Mild fibrosis (n = 16)	P
Age (yr)	56.47 ± 10.92	60.75 ± 6.50	52.19 ± 12.85	0.024
Sex (male:female)	15 : 17	8 : 8	7 : 9	0.723
AST (IU/L)	105.47 ± 56.18	119.31 ± 55.75	91.63 ± 54.82	0.167
ALT (IU/L)	163.03 ± 102.99	167.81 ± 102.15	158.25 ± 106.95	0.798
Iron (µg/dL)	155.07 ± 43.27	149.50 ± 33.00	160.27 ± 51.71	0.513
TIBC (µg/dL)	356.97 ± 48.38	351.50 ± 41.23	362.07 ± 55.18	0.566
Transferrin saturation (%)	43.63 ± 11.69	42.51 ± 7.85	44.68 ± 14.62	0.621
Ferritin (ng/mL)	291.19 ± 213.72	329.41 ± 222.08	255.53 ± 206.72	0.362
Increased serum iron store	14 (43.75%)	8 (50.00%)	6 (37.50%)	0.476
Positive hepatic iron stain	4 (12.50%)	1 (6.25%)	3 (18.75%)	0.600
Viral genotype (1:2)	22 : 6	12 : 2	10 : 4	0.648
Viral load (× 10 ⁶ copies/mL)	4.94 ± 6.26	6.39 ± 8.16	3.48 ± 3.22	0.231

CHC: Chronic hepatitis C; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; TIBC: Total iron binding capacity. Data are expressed as mean ± SD or patients number (percentage). Transferrin saturation was calculated as serum iron divided by TIBC × 100%. The increased serum iron store was defined by transferrin saturation > 50% and/or ferritin > upper normal limit.

RESULTS

Thirty-two patients fulfilling inclusion criteria were studied. The demographic and laboratory data of the patients are summarized in Table 1. The mean age of the 32 patients was 56.47 ± 10.92 year-old. Fourteen patients (43.75%) had increased serum iron stores and only four patients (12.5%) had positive hepatic iron stain. In the four patients, three patients were grade one and one patient was grade two on Perls' stain. Of 32 patients, 16 patients showed severe hepatic fibrosis (stages 3 or 4) and 16 patients had mild fibrosis (stages 0, 1 or 2) on histology.

The CHC patients with severe hepatic fibrosis were significantly older than the CHC patients with mild fibrosis (60.75 ± 6.50 *vs* 52.19 ± 12.85 year-old; *P* = 0.024). The other variables showed in Table 1, including gender, liver enzyme tests, serum iron indices, increased serum iron store, positive hepatic iron stain, viral load and genotype of HCV, were not significantly different between patients with severe and mild hepatic fibrosis. In multivariate logistic regression analysis, the age at biopsy was still an independent predictor of severe hepatic fibrosis (Odds ratio = 1.312; *P* = 0.035) (Table 2).

We stratified our data according to patient sex because women may have lower serum iron markers than men. All the serum iron indices and hepatic iron stain were not associated with severe hepatic fibrosis in men and women, respectively. Univariate analysis across grades of histological inflammation activity also did not show a significant association between inflammation activity and any of the serum iron indices or the presence of hepatic tissue iron, age, gender, liver enzyme tests, viral load and genotype of HCV (data not shown).

The positive hepatic iron stain was significantly associated with the values of ALT (*P* = 0.017) and all the

Table 2 Multivariate logistic regression analysis of independent predictors of severe hepatic fibrosis

Variable	Odds ratio	95% CI	P
Age	1.312	1.020-1.688	0.035
Male gender	14.138	0.835-239.266	0.066
Increased serum iron store	0.834	0.081-8.595	0.879
Positive hepatic iron stain	1.584	0.067-37.349	0.775
Viral load	1.412	0.923-2.161	0.112

CI: Confidence interval; ALT: Alanine aminotransferase. The increased serum iron store was defined by transferrin saturation > 50% and/or ferritin > upper normal limit. The ALT and viral genotype were eliminated in multivariate backward logistic regression analysis. We forcibly add the two factors increased serum iron store and positive hepatic iron stain into the analysis.

Table 3 Univariate analysis of demographic and laboratory data in relation to hepatic iron stain

Variable	Positive hepatic iron stain (n = 4)	Negative hepatic iron stain (n = 28)	P
Age (yr)	52.50 ± 9.04	57.04 ± 11.19	0.446
Sex (male:female)	3 : 1	12 : 16	0.319
AST (IU/L)	141.50 ± 99.94	100.32 ± 47.89	0.473
ALT (IU/L)	275.00 ± 172.18	147.04 ± 82.23	0.017
Iron (µg/dL)	210.50 ± 45.11	146.20 ± 36.55	0.019
Transferrin saturation (%)	62.34 ± 10.71	40.64 ± 8.80	0.003
Ferritin (ng/mL)	591.23 ± 119.70	243.19 ± 184.65	0.008

Data are expressed as mean ± SD. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase. Transferrin saturation was calculated as (the serum iron divided by the TIBC) × 100%.

Table 4 Correlations with ferritin levels among laboratory data, hepatic inflammation and fibrosis scores

Variable	ALT	Iron	Transferrin saturation	Inflammation score	Fibrosis score
Spearman coeff	0.531	0.467	0.556	-0.085	0.317
<i>P</i>	0.003	0.011	0.002	0.659	0.094

ALT: Alanine aminotransferase. Transferrin saturation was calculated as (the serum iron divided by the TIBC) × 100%.

three serum iron indices, including ferritin (*P* = 0.008), iron (*P* = 0.019) and transferrin saturation (*P* = 0.003) (Table 3). The ferritin level had significant correlation with the value of ALT, iron and transferrin saturation in Spearman correlation test (*P* = 0.003, 0.011 and 0.002, respectively). Nonetheless, no significant correlation was found between ferritin and grade of inflammation activity or stage of hepatic fibrosis severity (Table 4).

DISCUSSION

It has been recognized for more than 30 years that iron stores may be increased in alcoholic liver disease^[17]. In nonalcoholic steatohepatitis, 58% of patients has elevated serum iron indices and in some cases increased hepatic iron stores^[18]. In order to prevent the effect of

the potential confounding factors in hepatic iron stores, we carefully excluded patients that had alcohol abuse (ethanol consumption > 20 g/d), BMI over 27 kg/m², previous ribavirin therapy and multiple transfusions. The lower rate of positive hepatic iron stain (12.5%) may partly be due to the stringent selection criteria used in our study. In our previous study, we showed that the HFE mutations associated with hereditary hemochromatosis were infrequent in Taiwan and they may not contribute to iron accumulation in CHC patients even when serum iron overload was observed in more than one third of these patients^[19]. Therefore, we didn't exclude the few CHC patients with HFE mutations in our study.

Although the iron-related oxidative stress may play a role in the pathogenesis of CHC, the association between serum iron markers, hepatic iron stores, and hepatic fibrosis stage remains controversial. Previous studies had evaluated the potential impact of hepatic iron store on CHC but they had produced discordant results. Three studies had found that hepatic iron tissue deposition was associated with severe hepatic fibrosis in patients with CHC^[20-22]. Despite the association, they did not found a correlation between the amount of hepatic iron store and the fibrosis score. The absence of dosing effect suggests that there is a cut-off point at which all patients are more likely to have severe fibrosis, and all patients with values above this level have an equal risk regardless of the quantity of tissue iron concentration^[20]. In other words, there is a threshold effect, and once present, increasing hepatic iron does not correlate with increasing fibrosis^[22]. The other studies had proposed the discordant conclusions. No association was observed between the presence of hepatic iron deposition and fibrosis score in these reports^[23-26]. In our study, significant iron that was detectable histologically was also unrelated to the severity of hepatic fibrosis. It is well established that a heavy iron overload per se can cause hepatic fibrosis, as observed in patients with hereditary hemochromatosis. In a semi-quantitative evaluation of hepatic iron in patients with CHC, most had minimal or mild deposits^[25]. Our study had the similar results. In the four patients with positive hepatic iron stain, three patients were grade one and one patient was grade two on Perls' stain. This may be the reason why the hepatic iron stain was not associated with severe hepatic fibrosis in our study. Mild degree of hepatic iron deposition may not reach the threshold at which iron will enforce hepatic injury.

In the present study we did not find any association between serum iron indices or hepatic iron stain and degree of hepatic fibrosis or inflammation activity in patients with CHC. However, our study had showed that hepatic iron stain was associated with altered ALT values and serum iron indices. The ferritin levels also showed correlation with ALT values and the other two serum iron indices. That is, the biochemical injury of liver can be predicted by tissue or serum iron contents but the histological damage can't. This is consistent with the finding that the decline in serum AST and ALT values after phlebotomy is not associated with a change in histological activity of inflammation or fibrosis^[27]. The mechanism by which iron accumulates in some patients with CHC is

unclear. Whether this iron accumulation is cause or result of liver injury is unknown. Previous studies had reported a positive correlation between serum ferritin concentration and ALT level in patients with CHC^[1,28]. Since serum iron index correlated significantly with the value of ALT, it was likely that the excess iron could be related to its release from destroyed hepatocytes as a result of liver injury associated with HCV. This suggested that iron parameters in patients with CHC acted either as markers of the chronic inflammatory state or cytolytic liver activity but did not directly reflect the progression of hepatic fibrosis. Furthermore, the tissue iron contents did associate with the all serum iron indices in our study. In other words, ferritin, iron and transferrin saturation were all excellent predictors for presence of hepatic iron in patients with CHC.

Our study found that older age at biopsy was associated with severe hepatic fibrosis in patients with CHC. This suggested that hepatitis C infection may somehow become more fibrogenic with advancing host age. This is in accordance with previous studies showing that severity of HCV-related liver injury can be predicted by patient age^[23,24,29]. The mechanism underlying this association is unknown. The possible explanations might include immune factors, increased fibrogenesis, or decreased fibrolysis^[9]. The ability of hepatocytes to regenerate or the state of activated hepatic lipocytes alters with age and thus gives rise to increased fibrosis^[29]. Nonetheless, these speculations are unproven yet. Our data allow a conclusion that CHC will place an increasing burden on health care services in the next decades as the population with CHC ages.

Our study do have a potential limitation. In two large-scale studies, age at onset of infection had been identified as predictive factor of progression in CHC^[13,30]. Since the time of onset of infection derived from clinical history may not be reliable, we had omitted this variant in our study.

In conclusion, the severity of HCV-related liver injury is associated with patient age at biopsy. Significant iron deposition in the liver is uncommon in CHC patients. Both serum iron indices and hepatic iron deposition show correlation with serum indices of chronic liver disease but are not related to grade and stage of liver histology. The viral load and genotype of HCV are also not associated with hepatic fibrosis severity and inflammation activity. Our study conclusions suggest that patients with CHC should be treated as early as possible. Our findings do not support the role for iron depletion therapy by phlebotomy in patients with CHC, including those with elevated serum iron indices or positive hepatic iron stain.

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RAPID COMMUNICATION

Dynamical changing patterns of histological structure and ultrastructure of liver graft undergoing warm ischemia injury from non-heart-beating donor in rats

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Abstract

AIM: To investigate the histological and ultra-structural characteristics of liver graft during different of warm ischemia time (WIT) in rats and to predict the maximum limitation of liver graft to warm ischemia.

METHODS: The rats were randomized into 7 groups undergoing warm ischemia injury for 0, 10, 15, 20, 30, 45 and 60 min, respectively. All specimens having undergone warm ischemia injury were investigated dynamically by light and electron microscopy, and histochemistry staining. After orthotopic liver transplantation (OLT), the recovery of morphology of liver grafts after 6, 24 and 48 h was observed.

RESULTS: The donor liver from non-heart-beating donors (NHBD) underwent ischemia injury both in the warm ischemia period and in the reperfusion period. Morphological changes were positively related to warm ischemia injury in a time-dependent manner during the reperfusion period. The results demonstrated that different degrees of histocyte degeneration were observed when WIT was within 30 min, and became more severe with the prolongation of WIT, no obvious hepatocyte necrosis was noted in any specimen. In the group undergoing warm ischemia injury for 45 min, small focal necrosis occurred in the central area of hepatic lobule first. In the group undergoing warm ischemia injury for 60 min, patchy or diffused necrosis was observed and the area was gradually extended, while hepatic sinusoid endothelial cells were obviously swollen. Hepatic sinusoid was obstructed and microcirculation was in disorder.

CONCLUSION: The rat liver graft undergoing warm ischemia injury is in the reversible stage when the WIT is within 30 min. The 45 min WIT may be a critical point of rat liver graft to endure warm ischemia injury. When the WIT is over 60 min, the damage is irreversible.

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Key words: Liver transplantation; Warm ischemia injury; Morphological observation

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INTRODUCTION

In the past 40 years, liver transplantation has achieved a great success and has become the most effective method to treat the end-stage hepatic diseases. Liver transplantation is developing rapidly as a result of perfect perioperative care and widespread applications of potent immunosuppressants. However, there is an obvious problem of donor organ shortage, especially in countries where the "brain-death" cases have not been legitimated. At present, a high percentage of liver grafts comes from non-heart-beating donor (NHBD) in these countries. Under these circumstances, liver grafts unavoidably encounter a period of warm ischemia injury and undergo further injuries in preservation and reperfusion process^[1-3]. Poor quality of liver grafts is considered an important risk factor greatly reducing the liver transplantation effectiveness^[4-6]. Clinical practice suggests that the warm ischemia time (WIT) should not be longer than 5 min^[7], and 10 min of WIT may be the upper limit. According to this, many donor livers are useless, thus aggravating the problem of donor liver shortage. This study was to observe the changing patterns of histological structure and ultrastructure of liver graft undergoing warm ischemia injury.

MATERIALS AND METHODS

Animals and grouping

Two hundred and ten healthy male adult Sprague-Dawley (SD) rats weighing 250-300g (Experimental Animal Center at Sun Yat-Sen University) were used in the study. The mean weight of recipient rats was slightly heavier than that of donor rats. According to WIT, 210 SD rats were randomly divided into seven groups. The duration of WIT was 0, 10, 15, 20, 30, 45 and 60 min respectively. Forty-two SD rats (6 each group) were used for the observation of warm ischemia injury. The other 168 were divided into 7 subgroups. Orthotopic liver transplantation (OLT) was performed in each group according to the predetermined WIT, 12 as donors and 12 as recipients. Histological, histochemical and ultrastructural changes were observed 6, 24 and 48 h respectively after reperfusion. Specimens taken from the right hepatic lobe of rats were divided into 4 types, one for routine olefin sections after fixation in formalin solution, one for glycogen staining after fixation in Gendre solution, one for enzyme histochemical staining after quick freezing in liquid nitrogen and one for ultrathin sections.

Establishment of animal model

Warm ischemia injury was induced by clamping the basilar part of the heart and blocking the thoracic aorta of the donor animals after the donor rat received 0.2 mL heparin sodium solution (1250 U heparin sodium) *via* dorsum of penis vein to establish the non-heart-beating donor model. Then the liver graft was dissected. The liver was then perfused *in situ* through the abdominal aorta with 20 mL chilled lactic acid Ringer's solution (50 U/mL heparin sodium) and stored in a bath of cold lactate Ringer's solution before transplantation. Immediately prior to the portal vein clamping, orthotopic liver transplantation was performed as previously described^[8] with minor modifications^[9]. The cold ischemia time (CIT) was 50 ± 3.5 min and the anhepatic period was 20 ± 2.5 min.

Observation indexes and methods

Specimens were fixed in formalin solution, routine 4-6 μ m paraffin sections were stained with HE for light microscopy.

Specimens were disposed by the typical ultra-histology methods, and the sections were observed under transmission electron microscope and scanning electron microscope.

Hepatic glycogen was stained by PAS reaction after the fresh specimens were fixed in Gendre's solution for 6 h. Tetrazolium blue, PPDA, magnesium activation were respectively adopted to observe the activities of SDH, CO and ATPase on 5-6 μ m thick cryo-sections.

RESULTS

Observation under light microscope

Histological structure changed slightly when WIT was shorter than 30 min. Cytoplasm loosening, cell edema, focal vacuole degeneration were noted when WIT was over 30 min, especially in the lobule center area. Leukocyte

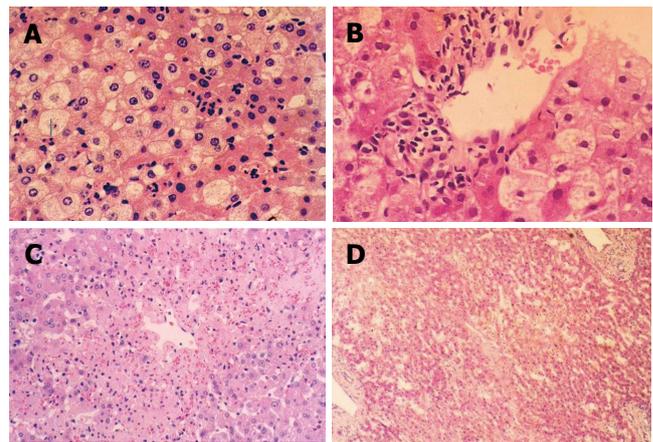


Figure 1 Cytoplasm loosening, cell edema, focal vacuole degeneration after reperfusion (10×10) in the group undergoing warm ischemia injury for < 30 min (A); obvious cell edema, ballooning-like degeneration after reperfusion (40×10) in the group undergoing warm ischemia injury for 30 min (B); focal necrosis around central lobule area after reperfusion (10×10) in the group undergoing warm ischemia injury for 45 min (C); plaque-like area necrosis after reperfusion (40×10) in the group undergoing warm ischemia injury for 60 min (D).

infiltration was noted in the portal area and acidophilus was obvious in some hepatocytes. The above pathologic changes aggravated when WIT was prolonged to 60 min. Cell degeneration was diffuse or extended to a focal area, even lipid degeneration could be seen. The degree of degeneration was dependent on the duration of WIT, but necrosis could hardly be observed under light microscope. After 6 and 24 h reperfusion, injury to liver graft became severer and hepatocytes presented with obvious edema and some ballooning degeneration in the group undergoing warm ischemia injury for 30 min (Figures 1A and B). Focal like necrosis could be noted in the lobule center area in the group undergoing warm ischemia injury for 45 min (Figure 1C), the change aggravated when WIT was prolonged. Forty-eight hours after reperfusion, hepatic injury resumed gradually in the group undergoing warm ischemia injury for < 45 min. Hepatocytes presented with plaque or diffused necrosis and the pathologic change was irreversible in the group undergoing warm ischemia injury for 50 min (Figure 1D).

Observation under electron microscope

The structure of mitochondria and endoresticule was normal when WIT was shorter than 15 min (Figure 2A). Mitochondria became swollen, density of basal plasma was reduced, endoresticule was enlarged and glycogen particles were reduced in the group undergoing warm ischemia injury for 30 min. Mitochondria crista became fuzzy and pale in the group undergoing warm ischemia injury for 45 min. While fuzzy or ruptured mitochondria crista, vacuole degeneration and broken endoresticule were noted in the group undergoing warm ischemia injury for 60 min. Six hours after reperfusion, damage to liver graft became severer and 24 h after reperfusion, mitochondria became swollen and basal density reduction was aggravated, but glycogen particles increased in the group undergoing warm ischemia injury for < 30 min (Figure 2B). Mitochondria became swollen, vacuole was

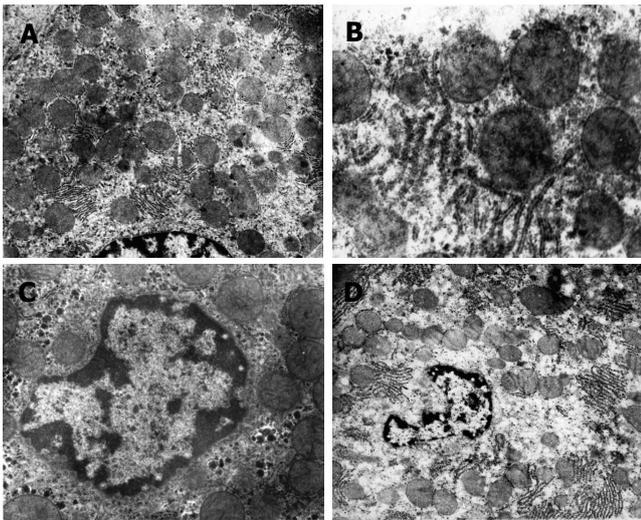


Figure 2 Swollen mitochondria, ruptured crista, rough endoplasmic reticulum degranulation ($\times 15000$) in the group undergoing warm ischemia injury for 30 min (A); phanero-swollen mitochondria and nuclear chromosome margination, and karyopyknosis after reperfusion ($\times 10000$) in the group undergoing warm ischemia injury for 45 min (B and C); significant swollen mitochondria, crista extinction, nuclear membrane rupture, karyolysis and karyorrhexis after reperfusion ($\times 5000$) in the group undergoing warm ischemia injury for 60 min (D).

degenerated, and rough endoreticule was broken, apoptosis of hepatic and endothelial cells was increased with chromosome margination, karyopyknosis and karyorrhexis in the group undergoing warm ischemia injury for > 30 min (Figures 2C and D). Endothelial gaps were enlarged, the sieve plate was mingled, some endothelial cells broke off, and therefore, some sinusoids were blocked with cytoplasmic bleb accumulation in the groups undergoing warm ischemia injury for 45 and 60 min (Figure 3A). Forty-eight hours after reperfusion, swollen mitochondria resumed gradually, glycogen particles increased obviously in the group undergoing warm ischemia injury for < 45 min. Mitochondria were extended with crista turbulence, vacuole, membrane rupture, and cell apoptosis and necrosis (with karyopyknosis, karyorrhexis and karyolysis) could be noted in the group undergoing warm ischemia injury for 60 min. The above changes were irreversible (Figure 3B). Most endothelial cells underwent necrosis and shedding, many hepatic sinusoids were full of cytoplasmic blebs, reticular fibrosis and hemocytes (Figure 3C). Under scanning electron microscope, endothelial cells presented with bleb or ballooning like degeneration, sinusoids were blocked, thus the microcirculation underwent irreversible disturbance (Figure 3D).

Histochemical observation

Our previous study has demonstrated that hepatic glycogen begins to reduce when WIT is prolonged to 30 min and the activities of SDH, CO and ATPase begin to decrease when WIT is longer than 45 min^[10]. The above changes were positively related to WIT in a time dependent manner during reperfusion in this study. PAS reaction and enzyme activities showed a recovering potency in the groups undergoing warm ischemia injury for 15 and 30 min. The liver graft underwent an irreversible injury and

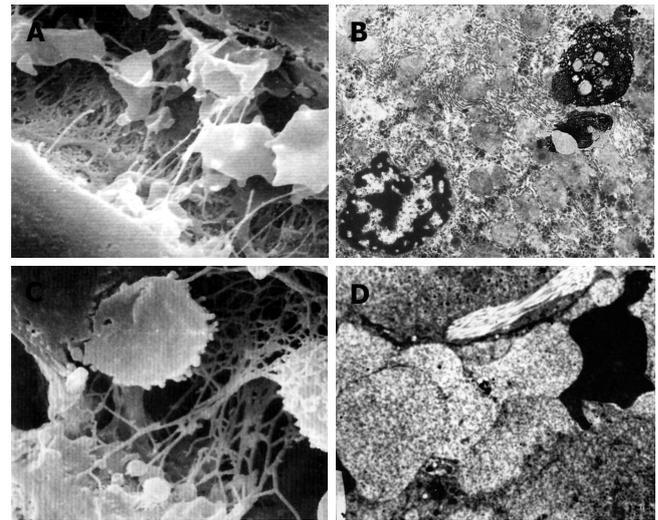


Figure 3 Cytoplasmic blebs and irregular endothelial sieve plate in some sinusoids after reperfusion (scanning electron microscope $\times 6000$) in the group undergoing warm ischemia injury for 45 min (A); apoptosis and necrosis of hepatocytes after reperfusion ($\times 8000$) in the group undergoing warm ischemia injury for 60 min (B); cytoplasmic blebs, reticular cellulose and hemocytes after reperfusion (scanning electron microscope $\times 6000$) in the group undergoing warm ischemia injury for 60 min (C); bleb or ballooning like swollen endothelial cells, blocked sinusoids and irreversible microcirculation disturbance after reperfusion ($\times 5000$) in the group undergoing warm ischemia injury for 60 min (D).

no evident recovery potency was found after implantation in the groups undergoing warm ischemia injury for 45 and 60 min.

DISCUSSION

How to evaluate the quality of liver grafts and how to ascertain the safety time limit for warm ischemia of liver grafts remain to be solved since warm ischemia injury affects the outcome of liver transplantation. Western transplantation community does not consider much of warm ischemia injury because their liver grafts are mainly taken from "brain-death" donors. Liver transplantation has become an effective management in the treatment of end-stage liver diseases, while the shortage of donor liver is a critical limiting factor for liver transplantation, thus people have begun to reconsider the marginal organ source like NHBD since 1990s^[11-13]. Some laboratory studies support a controversial 60 min WIT limitation^[14-16], but different experimental animals, warm ischemia models and experiment conditions may cause arguments about the WIT limitation. The argument about the WIT limitation has led to a worldwide investigation on warm ischemia-reperfusion injury^[17,18].

In the present study, we observed the changes of histological structure and ultrastructure of liver grafts during different WIT. Cellular edema and vacuole degeneration could be noted in warm ischemia period. The pathologic changes were aggravated with the prolongation of WIT, but necrosis was absent. Hepatocyte vacuole degeneration was due to swollen mitochondria and outstretched endoreticule. Glycogen-absorbed vacuole also could be seen. Under electron microscope, pathologic

changes were reversible, only part of cells underwent irreversible changes such as necrosis (with karyopyknosis, karyorrhexis and karyolysis) and apoptosis. However, liver grafts from NHBD underwent injuries both in the warm ischemia period and in the reperfusion stage. The degree of injury in the reperfusion stage was positively related to the duration of WIT. Histochemical observations showed that hepatic glycogen began to reduce when WIT was prolonged to 30 min. The activities of SDH, CO and ATPase began to decrease when WIT was longer than 45 min. The above changes were positively related to WIT in a time-dependent manner during the reperfusion period. PAS reaction and enzyme activities showed a recovering potency in the groups undergoing warm ischemia injury for 15 and 30 min. The liver graft underwent irreversible injury and no evident recovery potency was found after implantation in the groups undergoing warm ischemia injury for 45 and 60 min.

In conclusion, the pathologic changes of liver grafts undergoing only warm ischemia injury are reversible when WIT is shorter than 60 min, but the damage to liver graft would aggravate at the reperfusion stage, suggesting that rat liver grafts undergoing warm ischemia injury are in the reversible stage when WIT is within 30 min. The 45 min of WIT may be a critical point of rat liver grafts to tolerate warm ischemia injury and when WIT is prolonged to 60 min, the damage is irreversible.

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CASE REPORT

Alpha-1-antitrypsin deficiency resulting in a hitherto unseen presentation of hepatocellular carcinoma: Polycythemia but with normal alpha fetoprotein

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Abstract

Polycythemia is a known paraneoplastic manifestation of hepatoma, but only in the presence of alpha-fetoprotein (AFP). We present a case of polycythemia in the absence of AFP, and suggest concurrent alpha-1-antitrypsin deficiency as the cause for breaking this rule. We also suggest a reason for the apparent constant conjunction between polycythemia and AFP in hepatoma.

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Key words: Hepatoma; Polycythemia; Alpha 1 antitrypsin; Ephrin-A; Alpha-fetoprotein

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INTRODUCTION

Polycythemia is a known paraneoplastic manifestation of hepatoma, but only in the presence of alpha-fetoprotein (AFP). We present a case of polycythemia in the absence of AFP, and suggest concurrent alpha-1-antitrypsin deficiency as the cause for breaking this rule. We also suggest a reason for the apparent constant conjunction between polycythemia and AFP in hepatoma.

CASE REPORT

A 79-year-old man was referred for dizziness with a history of benign prostatic hypertrophy and cholecystectomy. He was taking finasteride and ranitidine. He was an ex-smoker with a forty-pack year history and had never drunk alcohol heavily. Clinical examination was unremarkable but for a smoothly enlarged liver. Full blood count revealed polycythemia (haemoglobin 176 g/L, haematocrit 0.527), with a normal platelet count and white cell count. Chest X ray and ECG were normal. Oxygen saturation was 96% on air.

Abdominal ultrasound revealed a normal spleen and a 47 mm × 57 mm × 49 mm mass in the right lobe of the liver posteriorly, which was compressing the inferior vena cava. A CT scan demonstrated a well-defined rounded mass in the hilum of the liver with a mixed attenuation pattern, consistent with either hepatocellular carcinoma (HCC) or haemangioma. Serum AFP [8 µg/L (normal 0-16)], liver function tests, and hepatitis serology were normal, except IgG antibodies to hepatitis A. MRI of the liver suggested HCC as a more likely diagnosis than haemangioma. With normal AFP and liver function tests, a biopsy of the lesion was carried out. The histology confirmed HCC with adjacent cirrhosis.

He was referred to a specialist centre and further imaging confirmed extra hepatic disease with intra-abdominal and mediastinal lymphadenopathy. There was local vascular invasion but no peritoneal dissemination. A further liver biopsy found underlying fibrosis and DPAS (diastase periodic acid Schiff) positive globular material in the hepatocytes, consistent with alpha-1-antitrypsin (AAT) deficiency. Because of vascular involvement he was deemed unsuitable for surgery and was entered into a trans-arterial embolisation versus chemoembolisation trial.

DISCUSSION

Hepatocellular carcinoma can present with various paraneoplastic manifestations including polycythemia, hypercholesterolemia, hypoglycemia and hypercalcemia^[1]. Our case is unique as it demonstrates an unreported phenomenon: HCC with polycythemia, but normal serum AFP. Polycythemia is strongly related to tumour burden and AFP, and is usually associated with markedly raised serum AFP levels^[2,3].

Polycythemia is only partly due to increased erythropoietin production, as raised serum erythropoietin can be present in up to 23% of HCC patients^[4], yet polycythemia is found only in approximately 1% of patients. This implies that erythropoietin production may be necessary, but is certainly not sufficient for polycythemia, and other factors must be implicated.

One such factor may be the expression of the erythropoietin receptor. This is upregulated by Ephrin-A1, a ligand for the Eph (erythropoietin producing hepatocellular) receptor tyrosine kinase^[5]. Ephrin-A1 expression upregulating the erythropoietin receptor and thus resulting in the appearance of polycythemia would explain the constant conjunction hitherto reported in HCC between polycythemia and AFP, as there is a strong correlation between the presence of AFP in HCC with the expression of Ephrin-A1, which is known to induce AFP^[5].

We therefore suggest that the association between polycythemia and raised AFP previously noted in HCC is because both arise from the expression of Ephrin-A1.

Our patient's normal AFP (despite his polycythemia) may be related to his AAT deficiency. Previous reports demonstrated high serum AFP levels in neonates with neonatal hepatitis, either idiopathic or due to extrahepatic biliary atresia. However, AFP is not raised in those infants with neonatal hepatitis and AAT deficiency. It was postulated that this is because alpha-1-antitrypsin is a rate limiting factor in the production of AFP^[6]. To our knowledge, the possibility of AAT deficiency resulting in normal AFP in HCC in adults has not been raised.

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CASE REPORT

Retinal vein thrombosis associated with pegylated-interferon and ribavirin combination therapy for chronic hepatitis C

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Abstract

An estimated 300 million people worldwide suffer from chronic hepatitis C with a prevalence of 0.8%-1.0% of the general population in Canada. An increasing pool of evidence exists supporting the use of pegylated-interferon (pegIFN) and ribavirin combination therapy for hepatitis C. We report a 49-year old male of North American aboriginal descent with chronic hepatitis C (genotype 2b). Biopsy confirmed that he had cirrhosis with a 2-wk history of left eye pain and decreased visual acuity. He developed retinal vein thrombosis after 16 of 24 wk of pegIFN- α 2a and ribavirin combination therapy. He was urgently referred to a retinal specialist and diagnosed with non-ischemic central retinal vein occlusion of the left eye. PegIFN and ribavirin combination therapy was discontinued and HCV RNA was undetectable after 16 wk of treatment. Hematologic investigations revealed that the patient was a factor V Leiden heterozygote with mildly decreased protein C activity. Our patient had a number of hypercoagulable risk factors, including factor V Leiden heterozygosity, cirrhosis, and hepatitis C that alone would have most likely remained clinically silent. We speculate that in the setting of pegIFN treatment, these risk factors may coalesce and cause the retinal vein thrombosis.

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Key words: Interferon; Pegylated-interferon; Hepatitis C; Cirrhosis; Retinal vein thrombosis; Thrombosis; Central retinal vein occlusion

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INTRODUCTION

An estimated 300 million people worldwide suffer from chronic hepatitis C with a prevalence of 0.8%-1.0% of the general population in Canada^[1]. In the last 10 years, dramatic advances have been made in the treatment of this common chronic condition. The pegylated-interferon (pegIFN) and ribavirin combination therapy has been shown to result in sustained virologic response rates of 46%-77%, depending on viral genotype^[2]. Evidence has also emerged regarding the utility of interferon in cirrhotic hepatitis C treatment with reduced rates of both hepatocellular carcinoma and improved survival^[3-5]. With the growing enthusiasm amongst patients and physicians alike, in favour of treatment as a result of the increasing pool of evidence supporting the use of interferon-based regimens, its adverse effects need to always be recognized and periodically reviewed.

Although interferon or pegIFN therapy can affect any organ system, the most commonly reported side effects include flu-like symptoms such as fever, chills, myalgia, fatigue, diarrhea, nausea and vomiting. Central nervous system disturbances including depression, suicidal ideation, confusion and mental status changes can occur, especially in patients with pre-existing histories. Hematologic side effects, including anemia, thrombocytopenia, and neutropenia, require ongoing monitoring. The reported withdrawal rates due to adverse effects, in studies examining interferon-based combinations are 7%-8%^[2,6].

We report a case of central retinal vein thrombosis in a cirrhotic hepatitis C patient during pegIFN and ribavirin combination treatment.

CASE REPORT

A 49-year old male of North American aboriginal descent, with chronic hepatitis C (genotype 2b) and biopsy confirmed cirrhosis, presented with a 2-wk history of left eye pain and decreased visual acuity, after a 16-24 wk course of therapy with pegIFN- α 2a at a dose of 180 μ g per week injected subcutaneously and 800 mg ribavirin per day (Pegasys, Hoffmann-La Roche, Mississauga, ON, Canada). His past medical and family histories were negative for any thrombophilia. Specifically, he had no

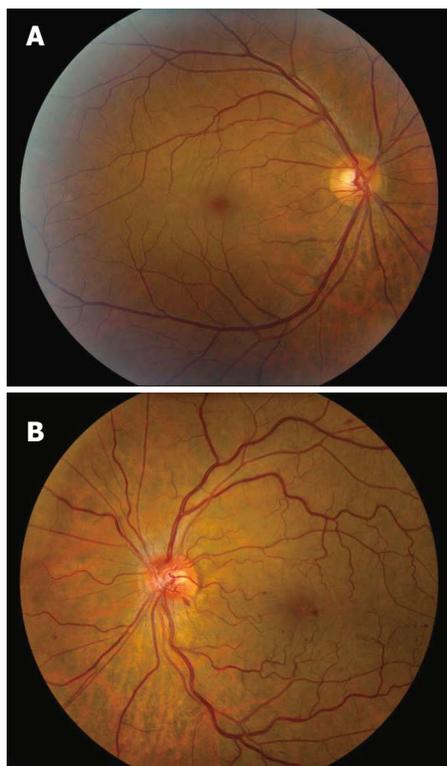


Figure 1 Normal fundus in the right eye (A) and marked venous tortuosity and scattered retinal hemorrhages in the left eye (B).

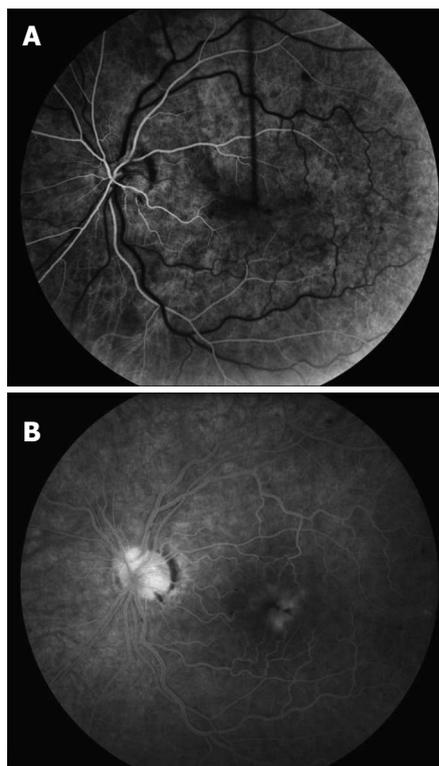


Figure 2 Left eye angiogram. A: Early phase image revealing delayed venous filling; B: Late phase image revealing leakage of fluorescein in the macula.

history of superficial or deep venous thrombosis and no history of thromboembolic events. Prior to the initiation of treatment, he had no evidence of decompensated liver disease and his serum alanine aminotransferase (ALT) was 256 U/L (upper limit of normal < 50U/L). Abdominal sonographic imaging revealed a cirrhotic liver with mild splenomegaly. Serial blood work performed at 4 and 8 wk of treatment revealed that his serum ALT levels were 67 U/L and 45 U/L (normal < 55 U/L), respectively. There were no complications associated with the treatment regimen prior to his presentation at 16 wk. One week following the onset of left eye pain and decreased visual acuity, he was assessed by an optometrist who prescribed eyeglasses. Due to the continued symptoms he presented to our hepatitis clinic, two weeks after the initial onset of symptoms. He was urgently referred to a retinal specialist and diagnosed with non-ischemic central retinal vein occlusion of the left eye (Figure 1). Fluorescein angiogram revealed delayed venous filling (Figure 2A) and associated macular edema (Figure 2B). Visual acuity at presentation was 20/20 in the right eye, and 20/70 in the left eye. PegIFN & ribavirin combination therapy was discontinued and the HCV RNA after 16 wk of treatment was undetectable.

Subsequent hematologic investigations to look for a hypercoagulable condition revealed heterozygosity for factor V Leiden, and a mildly decreased protein C activity at 0.49 U (lower limit of normal 0.65 U), confirmed by repeat testing. Antithrombin III and protein S levels were within normal limits but at the lower range with values of 0.72 U and 0.66 U, respectively (lower limits of normal for antithrombin III and protein S, 0.70 and 0.65 respectively). Cryoglobulins and the lupus anticoagulant were negative. Six months following the onset of our patient's left eye pain, his visual deficit remained unchanged.

DISCUSSION

Interferons comprise a group of pleiotropic proteins with anti-viral, anti-inflammatory, and anti-angiogenesis characteristics. Interferons are also multifunctional immunoregulatory cytokines with effects at various points in the cytokine cascade, likely accounting for their immunostimulatory effects^[7]. Due to their various mechanisms of action, interferons are well recognized to cause a variety of side effects as previously mentioned. From an ophthalmologic standpoint, there have been few reported cases of retinal vein thrombosis in patients treated with interferon or pegIFN. We are aware of only three reports in the medical literature^[8-10]. Of these, two of the reports describe this rare complication in cirrhotic patients being treated for hepatitis C^[9,10].

Regarding the potential thrombogenic properties of interferon, Guyer *et al*^[11] in a 1993 paper reporting retinopathy, have suggested that IFN therapy may cause immune complex deposition in the retinal vasculature and leukocyte infiltration, leading to retinal ischemia, congestion, and hemorrhage. Interferon therapy has also been reported to induce a number of thrombogenic autoantibodies, including cryoglobulins, anti-nuclear, anti-smooth muscle, anti-liver-kidney microsomal, anti-thyroglobulin, and anti-phospholipid antibodies, which are thought to play a role in the pathogenesis of a hypercoagulable state^[10].

In our patient, the causative role of pegylated-interferon therapy in inducing a hypercoagulable state that results in the retinal vein occlusion is strong given the temporal occurrence. However other risk factors may also have contributed. Considering the key role of the liver in coagulation, cirrhosis results in various impairments via multiple mechanisms: quantitative and qualitative

platelet defects, decreased production of coagulation and inhibitor factors, vitamin K deficiency, synthesis of abnormal clotting factors, decreased clearance of activated factors by the reticuloendothelial system, hyperfibrinolysis, and disseminated intravascular coagulation. The natural inhibitors of coagulation, antithrombin III, protein C and protein S, were at low to borderline levels of activity in our patient. Nevertheless, it is important to note that plasma activity of inhibitors between 50% and 70% alone, is not associated with increased thrombotic events in cirrhotics, possibly because of the proportional impairment of procoagulants^[12].

As part of the post diagnostic thrombophilic workup, our patient was found to be a heterozygote for the factor V Leiden mutation. Normally, factor V circulates in plasma as an inactive cofactor, awaiting activation by thrombin. Its inactivation requires protein C-mediated cleavage at arginine 306 and arginine 679. Genotypically, a point mutation in the gene-encoding factor V results in a missense mutation. The gene product, called factor V Leiden, which is not susceptible to cleavage by activated protein C, is inactivated more slowly and therefore confers an increased risk of venous thrombosis. The prevalence of heterozygosity for factor V Leiden is 5%-6% and is the most common inherited thrombophilia^[13]. The lifetime risk of thrombosis in heterozygotes compared to patients with no defect has been found to range 2.2-4.9^[14,15].

The hepatitis C virus itself has been found to induce a variety of potentially thrombogenic antibodies such as cryoglobulins, anti-nuclear, anti-smooth muscle, anti-cardiolipin and anti-phospholipid antibodies^[16]. Since the envelope proteins of cytomegalovirus and herpes viruses have been reported to function as a source of procoagulant phospholipid, one could speculate that the hepatitis C envelope could also have procoagulant activity^[17].

Our patient had a number of hypercoagulable risk factors that alone, most likely would have remained clinically silent. We speculate that in the setting of pegIFN treatment, these risk factors may coalesce and result in the retinal vein thrombosis. It is interesting that the three cases of retinal vein thrombosis described by Nadir *et al*^[10] were being actively treated for hepatitis C with pegIFN, but were also found to have primary defects contributing to a hypercoagulable state including protein S deficiency, hyperhomocysteinemia, heterozygosity for factor V Leiden, anti-phospholipid and anti-cardiolipin antibodies. Therefore we conclude that pegIFN treatment is an important risk factor for the development of retinal vein thrombosis, however based on our case and those described in the literature, other underlying risk factors may also need to be present. We emphasize that retinal vein thrombosis is still a rare complication, and we would not advocate the routine thrombophilic work-up of patients being considered for pegIFN treatment. However, the diagnosis needs to be considered in any patient on pegIFN presenting with decreased visual acuity or eye pain and any patient on pegIFN therapy presenting with manifestations of a thrombotic episode needs to undergo further hematologic investigation.

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Solitary pulmonary metastasis arising thirteen years after liver transplantation for HBV-related hepatocellular carcinoma

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Abstract

We described a 59-year-old male patient who underwent liver transplantation in 1989 for hepatocellular carcinoma (HCC) complicating hepatitis B virus (HBV) cirrhosis. In 2001 (12 years after liver transplantation), he developed a lung metastasis of HCC without intrahepatic recurrence and the resection was done. In July 2003, he was symptom free without any recurrence. HCC metastasis can develop even after a very long time of liver transplantation. Many HCCs grow slowly, and the growth rate of recurrent tumors in patients receiving immunosuppressive therapy is significantly greater than that of those who do not receive immunosuppressive therapy.

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Key words: Hepatitis B virus; Liver transplantation; Hepatocellular carcinoma; Metastasis; Immuno-suppression

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world and the third cause of cancer-related death^[1]. Cirrhosis mainly caused by hepatitis B and C viruses (HBV and HCV) constitutes the main risk factor for HCC with an yearly cumulative incidence of 3%^[1].

Liver transplantation is claimed to simultaneously cure the tumor and the underlying cirrhosis in selected patients. The 5-year survival rate can be achieved in 75% of optimal candidates for liver transplantation (single nodule < 5 cm or up to three nodules < 3 cm in diameter) with a recurrence rate below 15%^[1,2]. For patients with a single resectable HCC complicating cirrhosis, an alternative strategy is to offer resection first and then liver transplantation, if the tumor recurs or if the liver function deteriorates (salvage OLT)^[3].

However, the rate of HCC recurrence is high even after liver transplantation^[4]. The most frequent sites of recurrence of HCC after OLT are the lung (with a frequency of 51%) and the liver allograft (46%)^[4,5]. The great majority of cancer recurrences appear within 5 years of liver transplantation.

We described a 59-year-old male patient who underwent liver transplantation in 1989 for HCC complicating HBV cirrhosis. In 2001 (12 years after liver transplantation), he developed a lung metastasis of HCC without intrahepatic recurrence and the resection was done. In July 2003, he was symptom free without any recurrence.

CASE REPORT

A 59-year-old man developed deep fatigue and jaundice in May 1986. His serum alanine aminotransferase was 145 IU/L (normal < 40 IU/L) and tests for serum HBsAg, HBeAb, and HbeAb were positive, HBV-DNA was undetectable by hybridization assay. A liver biopsy showed cirrhosis. Serum level of alpha-feto-protein was 200 ng/mL (normal ≤ 15 ng/mL). Imaging studies (computerized tomography and ultrasound) showed a nodule (2 cm in diameter) in segment V with typical features of HCC.

In February 1987, segment V was resected. Histological examination confirmed a 2-cm nodule of HCC with a pseudo-capsule characterized by a sclerotic tissue with an intravascular extension of the tumor. After the intervention, alpha-feto-protein decreased to 20 ng/mL.

In December 1988, alpha-feto-protein raised to 100 ng/mL. Ultrasound and arteriography revealed nodules in the segments IV and VIII. The patient received three cures of chemoembolization (from December 1988 to March 1989) and 3 MU interferon thrice a week for 3 mo until liver transplantation, in order to make serum HBV-DNA negative. In August 1989, orthotopic liver transplantation was performed. Histological examination of the implanted liver showed micronodular cirrhosis without any evidence of neoplasia. He was given cyclosporin (18 mL/d),

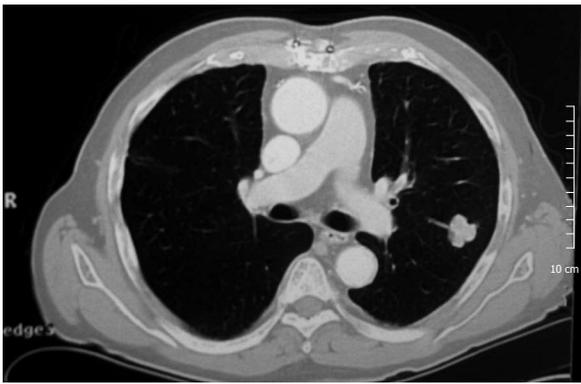


Figure 1 A 2-cm polylobulated and homogeneously dense lung nodule in the upper left lobe with no calcifications.

prednisone (20 mg/d) and azathioprine (150 mg/d). During the following 2 mo (October 1989), he was treated with cyclosporin (10 mL/d), prednisone (15 mg/d), and azathioprine (50 mg/d). During the following 14 mo (October 1990), he was treated with cyclosporin (0.6 mL/d), prednisone (10 mg/d), and azathioprine (50 mg/d).

During the following years, he had a 4-mo evaluation. Transaminase, alpha-feto-protein and imaging studies (ultrasound and thoraco-abdominal CT) did not show any sign or symptom of HCC recurrence. Rejection and opportunistic infections did not occur. He did not receive HBsAb immunoglobulins and had no reactivation of HBV infection.

In July 2001 (143 mo after liver transplantation), CT scan revealed a 2-cm isolated nodule in the upper left lung lobe, which was polylobulated and homogeneous without calcification (Figure 1). Alpha-feto-protein increased for the first time to 105 ng/mL. He was treated with prednisone (4 mg/d) and azathioprine (50 mg/d). In May 2002 (153 mo after liver transplantation), an atypical resection of the upper left lobe was performed. Histological examination showed poorly differentiated HCC metastasis. After the intervention, alpha-feto-protein returned to its normal level (3.5 µg). Repeated biochemical and imaging examinations did not show any recurrence in the lung, liver or other organs.

At the last follow-up visit (167 mo after liver transplantation and 14 mo after lung resection), he was symptom free. Thoraco-abdominal CT scan did not show any recurrence (July 2003) and serum alpha-feto-protein was 2 ng/mL (normal \leq 15 ng/mL).

DISCUSSION

Recombinant interferon- α (IFN- α) treatment prior to liver transplantation does not seem to reduce the rate of HBV infection; a residual infectivity may persist even in the absence of detectable serum HBV-DNA by standard method and its role in the carcinogenetic process cannot be eliminated^[6].

The 5-year survival rate can be achieved in 75% of optimal candidates for liver transplantation (early HCC, single nodule < 5 cm or up to three nodules < 3 cm in diameter) with a recurrence-free survival rate of

Table 1 HCC recurrence after liver transplantation

	Number of patients	Recurrence (%)	Pulmonary metastasis (%)	Mean delay (mo)
Yokoyama, 1991 ^[9]	100	43 (43/100)	12 (12/100)	34
Ferris, 1996 ^[4]	124	28 (35/124)	14 (18/124)	18
Wallis March, 1997 ^[10]	214	40 (71/214)	11 (23/214)	24
Total	438	34 (149/438)	12 (53/438)	25

92%^[7]. Most recurrent tumors arise during the first 2 years after resection, which might be explained by the multicentric nature of HCC in cirrhotic livers rather than by intrahepatic metastasis. The multifocal nature of HCC was examined in the livers from patients undergoing liver transplantation. Certain histological findings in the implant, such as the presence of capsular and microvascular invasion, are considered as signs of a more aggressive tumor associated with a greater incidence of recurrence^[8].

When recurrence after liver transplantation occurs, the most frequent sites are lungs (51%), liver allograft (46%), and lymph nodes (43%). Ferris *et al*^[4] have reported a mean interval of 18 mo.

In the three major studies^[4,9,10] involving HCC, the recurrence rate is 34% in 438 patients after liver transplantation (Table 1).

In a great majority of cases, recurrence of HCC occurs within 5 years of liver transplantation. The longest time to recurrence described in the literature is 124 mo^[4]. To our knowledge, the time between liver transplantation and recurrence is the longest in our case (12 years, 143 mo).

An active approach to the management of resectable pulmonary metastasis from HCC is justified in selected patients, which can permit a prolonged survival^[11]. Most pulmonary metastases of HCC are multiple and not amenable to surgical resection. If any solitary pulmonary metastasis encountered is resectable, the patient should undergo surgery.

The selection of patients with early HCC is the main factor affecting HCC recurrence after liver transplantation. At this early stage of tumor development, there are no other factors that have prognostic values. In patients from Western countries, the progression of HCC is usually slow and is related to tumor size^[2]. The mechanism for late recurrence of HCC remains unclear and some hypotheses have been proposed such as intraoperative surgical manipulation^[12], embolization of tumor cells via the hepatic veins before or during liver transplantation, which can result in the trapping of micrometastasis within the capillary network of the lungs and immunosuppressive therapy potentiating macroscopic growth of nodules^[5].

The effect of long-term immunosuppressive therapy on tumor growth in patients with HCC is unknown. It has been suggested that while many HCCs are growing slowly, the growth rate of recurrent tumors in patients receiving immunosuppressive therapy is significantly greater than that in those who do not receive immunosuppressive therapy, indicating that immunosuppressive therapy plays a major role in tumor recurrence after liver transplantation^[9].

In fact, it has been shown that the risk of recurrence in patients who continue to receive corticosteroids may be as much as four times higher than that in patients who stop receiving corticosteroids soon after the liver transplantation^[2,6].

In our case, immunosuppressive therapy seemed to be well balanced, because the patient had neither rejection nor opportunistic infections.

In conclusion, HCC metastasis can develop even after a long time of liver transplantation. A systematic long-term follow-up is necessary. In case of single lung metastasis without any other localization, it is possible to resect it, allowing to prolong the survival of the patient. It is very important to maintain vigilance after liver transplantation, because the risk of recurrence exists for a long time after liver transplantation.

Immunosuppressive therapy, one of the possible key factors in controlling the response to neoplasm, can reduce the treatment time^[13] and achieve immunologic tolerance and reduce the use of immunosuppressive drugs^[12].

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CASE REPORT

Biliary tuberculosis causing cicatricial stenosis after oral anti-tuberculosis therapy

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Abstract

A 36-year-old Philippine woman presented with dark urine and yellow sclera. Endoscopic retrograde cholangiopancreatography (ERCP) confirmed dilatation of the intrahepatic bile ducts and also showed an irregular stricture of the common hepatic duct at the liver hilum. Histological examination of biopsies from the bile duct revealed epithelioid cell granulomas and caseous necrosis. Tubercle bacilli were then detected on polymerase chain reaction (PCR) testing of the bile, giving the diagnosis of biliary tuberculosis. Although microbiological cure was confirmed, the patient developed cicatricial stenosis of the hepatic duct. She underwent repeated treatments with endoscopic biliary drainage (EBD) tubes and percutaneous transhepatic biliary drainage (PTBD) tubes, and the stenosis was corrected after 6 years. We present a case of tuberculous biliary stricture, a condition that requires careful differentiation from the more common malignancies and needs long-term follow-up due to the risk of post-treatment cicatricial stenosis, although it is rare.

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Key words: Biliary tuberculosis; Obstructive jaundice; Cicatricial stenosis; Polymerase chain reaction

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INTRODUCTION

The more common benign causes of biliary stenosis are

postoperative cicatricial stenosis and complications of chronic pancreatitis, duodenal papillitis, and congenital biliary dilatation, whereas tuberculous lesions, such as tuberculosis (TB) of the biliary lymph nodes, pancreatic TB, and biliary TB are rare. In this paper, we report a case of biliary TB causing obstructive jaundice and cicatricial stenosis after oral anti-tuberculosis therapy.

CASE REPORT

The patient was a 33-year-old female of Philippine origin. She presented with dark urine, yellow sclera, and malaise. She had lived in Japan for 3 years when she was admitted to our hospital. Her father and brother had a past history of pulmonary TB. She received no past treatment for TB. When she visited a local doctor in May 1998 for symptoms of dark urine and yellow sclera, she was found to have mild hepatic dysfunction and was thus referred to our hospital with suspected acute hepatitis. Viral, drug-induced, and auto-immune hepatitis were excluded, and she was treated with watchful anticipation as an outpatient. Abdominal ultrasound then revealed dilatation of the intrahepatic bile ducts and multiple intrahepatic hypodense areas, and the patient was admitted to our hospital for further investigation in February 1999. Admission findings included: height 154 cm, weight 54 kg, and body temperature 36.4°C. Her blood pressure was 112/62 mmHg, heart rate was 64 beats/min, and she had a sinus rhythm. Conjunctiva was not anemic or jaundiced. No superficial lymph nodes were palpable. Abdomen was flat and soft. The liver, spleen or masses were not palpable without abdominal pain or tenderness. Full blood examination revealed that she had mild anemia (117 mg/L; normal: 125-170 mg/L) and an elevated erythrocyte sedimentation rate (66 mm/h; normal: <10 mm/h). Serum biochemistry showed elevated biliary enzyme γ -glutamyltranspeptidase (201 IU/L; normal: 12-70 IU/L). Tumor markers CA19-9 (100 KU/L; normal: <37 KU/L) and PIVKA-II (43 AU/L; normal: <10 AU/L) were elevated. Abdominal ultrasonography (US) showed the hepatic parenchyma to be uniform and slightly hypertrophic, with dilatation of the intrahepatic ducts and multiple hypoechoic masses (Figures 1A and B). Abdominal computed tomography (CT) scans confirmed intrahepatic ductal dilatation and multiple hypodense lesions in the liver, some with microcalcifications. The early contrast phase images showed slight enhancement of the periphery of the lesions, while the late phase images showed uneven enhancement

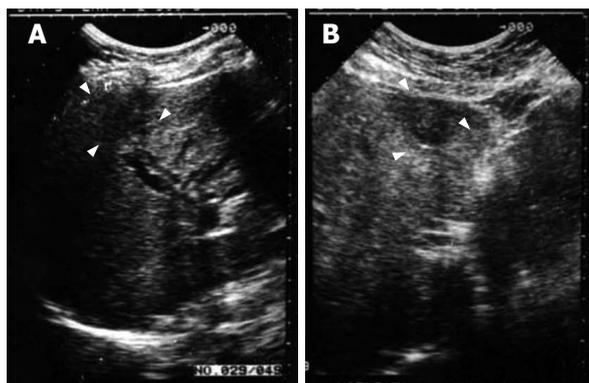


Figure 1 Ultrasonography findings of the liver. **A:** Dilatation of the intrahepatic ducts and a hypoechoic mass in the right lower anterior segment; **B:** A heterogeneous mass in the inner left segment.

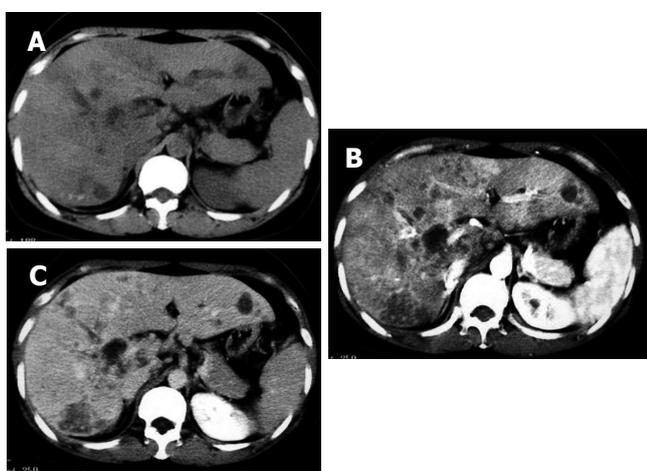


Figure 2 Abdominal computed tomography (CT). **A:** Plain CT images show intrahepatic ductal dilatation, micro-calcifications, and multiple hypodense lesions in the liver; **B:** Contrast CT image (early phase) shows clearly delineated hypodense lesions; **C:** Late phase CT image shows slightly enhanced hypodense lesions.

of the peripheral and central areas. Lymphadenopathy was seen both at the liver hilum and at the origin of the splenic artery (Figures 2A-C). Intraductal ultrasonography (IDUS) showed soft tissue masses at the liver hilum of the hepatic duct (Figure 3A), and circumferential thickening of the common hepatic duct (Figure 3B). Endoscopic retrograde cholangiopancreatography (ERCP) revealed that the common hepatic duct was narrowed over a 2 cm section, and the hepatic ducts were clumped and irregular at the liver hilum, with strictures of the feeding branches from each section of the liver (Figures 4A and B). Histopathological examination of endoscopic biopsy specimens from the common hepatic duct at the liver hilum revealed granulomas with epithelioid cells (Figure 5A), whereas a biopsy specimen from a hepatic mass showed very mild atrophy and marked dilatation of the hepatic sinuses, with large foci of caseous necrosis surrounded by epithelioid granuloma (Figure 5B). Repeated bile cytodiagnosis showed no malignancy. Cholangiography showed irregular strictures of the intra and extra hepatic biliary ducts. So hepatic secondaries from malignant neoplasia were mostly suspected, but the

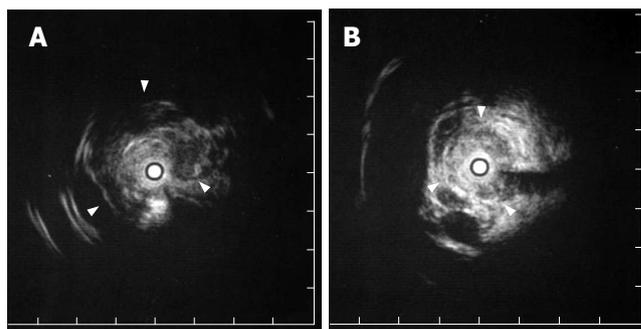


Figure 3 Endoscopic ultrasonography (EUS). **A:** Soft tissue masses at the liver hilum of the hepatic duct; **B:** Circumferential thickening of the common hepatic duct.

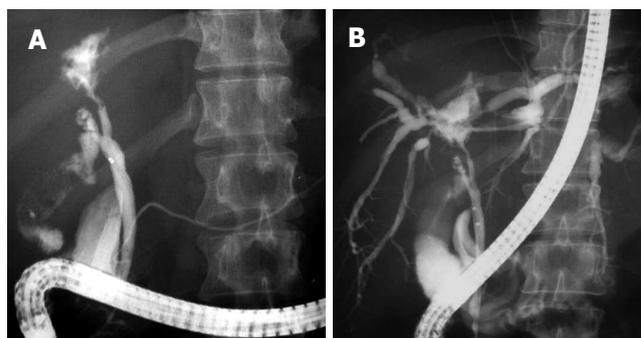


Figure 4 Endoscopic retrograde cholangiopancreatography examination. **A:** The common hepatic bile duct was narrowed over a 2 cm section, and strictures and irregularities of the hepatic bile duct at the liver hilum were revealed; **B:** The hepatic ducts at the liver hilum were clumped with strictures of the feeding branches from each section of the liver.

biopsies and bile cytodiagnosis did not show malignancy. Then the differential diagnosis could include primary biliary sclerosis (PSC), drug-induced cholestasis, and HIV-associated cholangiopathy. Serum ALP was normal with no obvious elevations in liver enzymes. Also serum anti-mitochondrial antibody and peripheral anti-neutrophil cytoplasmic antibody (pANCA), and smooth-muscle antibody did not elevate. Symptoms of inflammatory bowel disease, particularly ulcerative colitis did not present. She took no medicine and her serum HIV was negative. The biopsy findings of caseous necrosis and epithelioid granulomas, and bile polymerase chain reaction (PCR)-confirmed tubercle bacilli, led to the diagnosis of biliary TB. Tuberculin test was also strongly positive. Triple anti-tuberculosis therapy, comprising 400 mg isoniazid (INH) daily, 750 mg ethambutol (EB) daily, and 450 mg rifampicin (RIF) daily, was administered for 7 mo. Microbiological cure was confirmed in October 1999, with phlegm, gastric juice, bile, and feces negative for *Mycobacterium tuberculosis*. In December 2000, 14 mo after the completion of anti-tuberculosis treatment, the patient became febrile and jaundiced. Endoscopic retrograde cholangiography (ERC) demonstrated cicatricial stenosis of the common hepatic duct at the liver hilum. Because of the tight stricture at the liver hilum, and narrowing of many intrahepatic bile ducts, transpapillary stent placement was abandoned, and percutaneous transhepatic biliary drainage (PTBD) was performed instead (Figure 6A). Although she subsequently

Table 1 Summary of the 16 previous cases and our case of tubercular biliary stricture

No.	Age	Sex	Site of stricture	Initial presentation	Confirmation of diagnosis	Treatment	Outcome	Reference/Nation
1	30	M	CBD	CCC	Laparotomy frozen section	T-tube		Gupta <i>et al</i> ^[2] /India
2	78	F	Multiple	Bacterial cholangitis	Laparotomy frozen section	Laparoscopic cholecystectomy	Died of sepsis	Abascal <i>et al</i> ^[3] /Spain
3	46	F	CHD	CCC	Laparotomy frozen section	PTBD, surgical bypass was abandoned	Post anti-TB therapy, pulmonary calcification	Fan <i>et al</i> ^[4] /Hong Kong, China
4	38	M	CBD	CCC	Laparotomy frozen section	T-tube		Ratanarapee <i>et al</i> ^[5] /Thai
5	46	F	CHD		Bile cytology	EBD (Pl, metal)	Biliary stones, restenosis	Bearer <i>et al</i> ^[6] /USA
6	40	M	CBD	CCC	Laparotomy frozen section	Hepaticojejunostomy		Behera <i>et al</i> ^[7] /India
7	45	F	CBD	CCC	Laparotomy frozen section	Hepaticojejunostomy		Valeja <i>et al</i> ^[8] /India
8	70	M	CBD, CHD	CCC	Culture of biopsy of inguinal lymph node	ERBD (refused operation)	Post anti-TB therapy, pulmonary calcification	Hickey <i>et al</i> ^[9] /Ireland
9	46	M	CBD		CT guided FNAB	EBD	Restenosis	Kok <i>et al</i> ^[10] /Brunei
10	29	F	CHD, HD		Bile cytology	Left cholangiojejunostomy		Kok <i>et al</i> /Brunei
11	60	F	CBD	CCC	Laparotomy frozen section	Open biliary stenting		Kok <i>et al</i> /Brunei
12	44	F	CHD	CCC	Laparotomy frozen section	Hepaticojejunostomy	Hepatic calcification	Kok <i>et al</i> /Brunei
13	33	F	CHD	CCC	Laparotomy frozen section	Hepaticojejunostomy		Yea <i>et al</i> ^[11] /Taiwan, China
14	70	M	HD		PCR of bile	PTBD	Billroth II reconstruction	Yea <i>et al</i> /Taiwan, China
15	58	M	Multiple	CCC	Tissue obtained via PTBD	PTBD (metal)	Beaded type	Inal <i>et al</i> ^[12] /Turkey
16	66	M	CBD, RHD	CCC	Laparotomy frozen section	T-tube, PTCD	Post anti TB therapy	Prasad <i>et al</i> ^[13] /India
17	33	F	CHD		PCR of bile	PTBD, EBD	Pulmonary calcification, biliary stones, restenosis	Our case/Japan

CCC: Cholangio cell carcinoma; HD: Hepatic duct, RHD: Right hepatic duct; LHD: Left hepatic duct; CHD: Common hepatic duct; CBD: Common bile duct; FNAB: Fine-needle aspiration biopsy; ERCP: Endoscopic retrograde cholangiopancreatography; PTCD: Percutaneous transhepatic biliary drainage; EBD: Endoscopic biliary drainage.

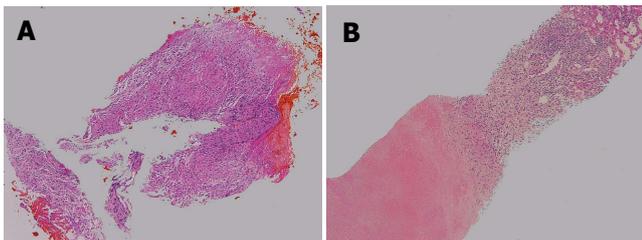


Figure 5 Histological findings of the biopsy specimen stained with HE. **A:** Photomicrograph of an endoscopic biopsy specimen from the common hepatic duct showing granulomas with epithelioid cells; **B:** Photomicrograph of a biopsy specimen from a hypochoic mass in the liver showing focal caseous necrosis surrounded by granuloma.

experienced repeated bouts of pyrexia and jaundice due to ascending cholangitis, the stenosis improved gradually, and fistularisation was achieved in July 2001 (Figure 6B). By February 2003, only two 7 Fr pigtail catheters were required for endoscopic biliary drainage (EBD) tube placement (Figure 6C). Two years later (in January 2005), a further bout of cholangitis made the patient febrile but not jaundiced, and no stenoses were detected on ERC (Figure 6D), so the EBD tubes were removed. She developed biliary stones in April of the same year, which was not detected before. However, EBD tube was reinserted and follow-up was continued at the time of writing this paper.

DISCUSSION

Benign biliary strictures fall into two etiological groups: traumatic (post operative, blunt, or penetrating injury) and nontraumatic (sclerosing cholangitis, recurrent pyogenic cholangitis, chronic pancreatitis, Mirizzi syndrome). The site and number of strictures depend on the cause. TB is

a rare cause of biliary obstruction. Hepatobiliary TB may be caused by three ways: spread of caseous material from the portal tracts into the bile ducts (most often), secondary inflammation-related tuberculous periportal adenitis, and spread of caseous material through the ampulla of Vater and ascending along the common bile duct. Hepatobiliary TB can be classified into 3 types: miliary hepatic TB, hepatic tuberculoma, and biliary TB^[1]. The majority are the miliary TB type. Hepatic tuberculoma requiring differentiation from hepatoma is relatively rare. Biliary TB is even more uncommon, and no cases of biliary TB causing obstructive jaundice due to biliary stenosis have been reported in Japan. A Pub Med search of papers published after 1985 has yielded 16 reported cases of biliary TB causing obstructive jaundice^[2-13] (Table 1). In each case, irregular stenosis of one or more bile ducts was seen on ERC, these findings differing considerably from those in cases of TB of the biliary lymph nodes or pancreatic TB, where obstructive jaundice is caused by extramural compression of the common bile duct (CBD). Differentiation from malignant neoplasia was often extremely difficult, and in 11 of the 16 cases laparotomy was performed without having excluded malignancy, and a preoperative diagnosis of TB was achieved through biopsy or PCR in only 5 cases. In 1 case, although the diagnosis of TB had been made, choledochoduodenostomy was required due to multiple strictures. 2 cases were complicated by biliary stones, and cicatricial restenosis occurred in the same cases following medical treatment. The bile duct might have been severely damaged by repeated inflammatory reactions and have become irreversibly scarred. One case had a postinflammatory stricture for nearly 2 years^[6] and one case required stent changes every 6 mo at the issue^[10]. And only 2 cases were

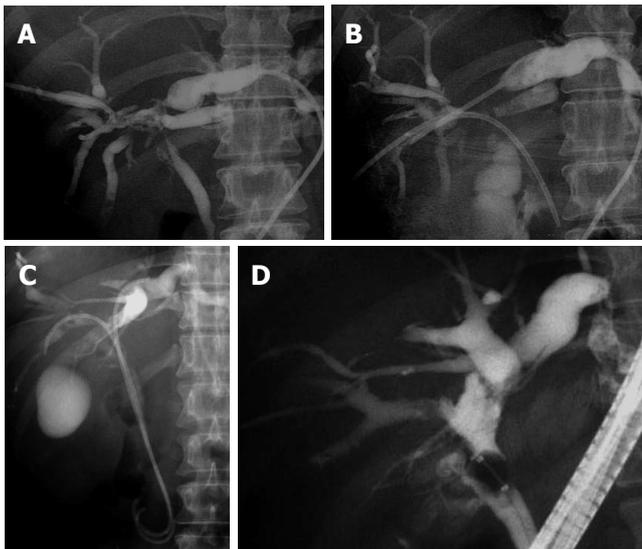


Figure 6 ERC following anti-tuberculosis therapy showing marked irregularity of the hepatic ducts and strictures of common hepatic duct. **A:** PTBD tubes were inserted via the B8 and B3 branches; **B:** The PTBD tube from the B5 branch was inserted into the common bile duct; **C:** EBD tubes were inserted into both hepatic lobes; **D:** The strictures of the hepatic ducts were recanalized.

with radiological evidence of pulmonary tuberculosis and one case with hepatic calcification, so TB must be considered in the differential diagnosis of any bile duct obstruction, particularly in patients from areas where TB is prevalent.

Biliary TB is a condition with no specific clinical findings and is usually diagnosed through biopsy or the detection of tubercle bacilli. The detection rate through culture is 0%-10%^[14]. However, even if epithelioid granulomas are identified, differentiation from conditions such as hepatic sarcoidosis or inflammatory bowel disease is important. Sarcoid granulomas are similar to TB granulomas, although in the former foreign body-type giant cells are seen in addition to Langhans giant cells, and foci of necrosis are rarely seen. In this case, acid-fast bacilli were not detected by culture or microscopy, and *Mycobacterium tuberculosis* was only detected through PCR testing of the bile. Since PCR testing for *Mycobacterium tuberculosis* is extremely sensitive,

it should be used extensively. Although favourable results can be achieved by medical therapy with repeated stenting of the bile ducts, long-term follow-up is required due to the risk of post-treatment cicatricial stenosis.

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CASE REPORT

A case of mucin producing liver metastases with intrabiliary extension

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Abstract

A 75-year-old man was admitted to our hospital with a diagnosis of liver metastases from colon cancer. He underwent right hemicolectomy for cecal cancer eight years ago, and had a metastatic liver tumor in segment 8 (S8), which was surgically resected about 4 years after the initial operation. Histopathological examination of the resected specimens from both operations revealed a well-differentiated adenocarcinoma with mucinous carcinoma. Four months after the second operation, computed tomography demonstrated a low-density lesion at the cut surface of the remnant liver. Although it was considered to be a postoperative collection of inflammatory fluid, it formed a cystic configuration and increased in size to approximately 5 cm in diameter. With a tentative diagnosis of a recurrence of metastatic cancer, partial hepatectomy of S8 was performed. Histological examination of the resected specimens also revealed mucinous adenocarcinoma, which had invaded into the biliary ducts, replacing and extending along its epithelium. Immunohistochemically, the tumor cells were positive for cytokeratin (CK) 20, but negative for CK7. Therefore, the tumor was diagnosed as a metastatic adenocarcinoma from colonic cancer. Liver metastases of colorectal adenocarcinoma sometimes invade the Glisson's triad and grow along the biliary ducts.

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Key words: Liver metastases; Mucin; Intrabiliary extension; Cytokeratin 7; Cytokeratin 20

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mucin producing liver metastases with intrabiliary extension. *World J Gastroenterol* 2006; 12(30): 4918-4921

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INTRODUCTION

Liver metastases can occur in 25%-35% of patients with colorectal carcinoma^[1], and surgical resection has become accepted as a reasonable treatment for patients with liver metastases. However, because of their propensity to spread along epithelial surfaces, liver metastases of colorectal carcinoma sometimes invade the Glisson's triad^[2]. Therefore, in contrast to hepatocellular carcinoma (HCC), bile duct invasions of liver metastases from colorectal carcinoma are seen in about 10%-12% of resected liver metastases^[3,4]. On the other hand, cystic formation caused by mucin producing metastatic tumor from colorectal cancer has only rarely been reported. Here, we report a case of mucin producing liver metastases with intrabiliary extension that was difficult to distinguish from benign cystic change.

CASE REPORT

A 75-year-old man was admitted to our hospital with a liver tumor. He underwent right hemicolectomy following a diagnosis of cecal cancer in January, 1994. Histological examination of the resected specimens revealed a well-differentiated adenocarcinoma with mucinous carcinoma, which invaded the subserosal layer and metastasized to regional lymph nodes. Four years after his initial operation, he underwent partial hepatectomy of segment 8 (S8) due to a diagnosis of metastatic liver tumor on June 22, 1998. Histopathological examination of the resected specimens revealed a well-differentiated adenocarcinoma with mucinous carcinoma, similar to cecal cancer. The tumor invaded only the bile duct, but surgical margin was negative for cancer. Four months after his second operation, abdominal computed tomography (CT) revealed a low-density lesion at the cut surface of the liver. Initially, it was considered to be a postoperative collection of inflammatory fluid. Through the continuous observation using periodical CT scans, the low-density lesion gradually formed a cystic mass over two years, but little change was found in size. Two years later, the mass increased in size

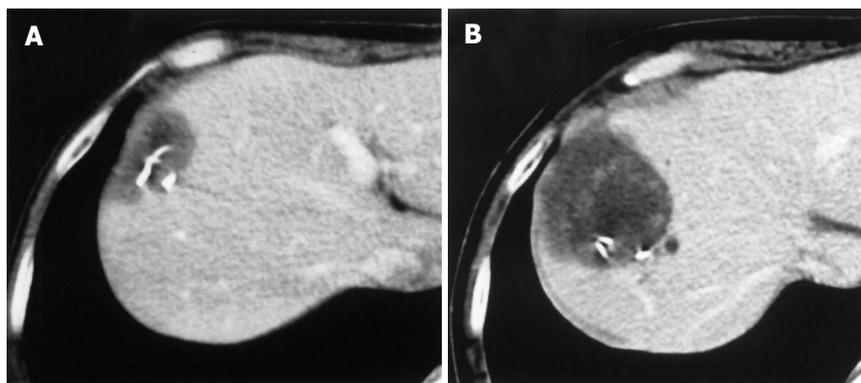


Figure 1 A low-density lesion at the cut surface of the liver in December, 1998 (A) and two years later, the mass increased in size to approximately 5 cm in diameter (B).



Figure 2 A low-density cystic tumor in S8 with local dilatation of the IHBD (arrows). The tumor was bordered by the diaphragm (arrow heads).



Figure 3 A low intensity signal on T1-weighted (A) and a high intensity signal on T2-weighted (B) images. The edge of the tumor was slightly enhanced by gadolinium, while the inside was heterogeneous (C).

to approximately 5 cm in diameter (Figure 1). Cytological examination from ultrasonography (US)-guided fine needle aspiration biopsy revealed it to be class III, indicating a borderline malignancy.

On admission, the patient exhibited no abnormal findings upon physical examination. Complete blood counts and serum chemistries were within normal limits. Among tumor markers, serum carcinoembryonic antigen (CEA) levels and alpha-fetoprotein (AFP) levels were within normal limits. The indocyanin green retention rate after 15 min (ICG R15) was 16.0%. Abdominal CT revealed a low-density cystic tumor of S8 that measured about 4 cm × 5 cm in size, with local dilatation of intrahepatic bile ducts (IHBD). The tumor was bordered by the diaphragm (Figure 2). Abdominal magnetic resonance imaging (MRI) revealed a tumor with a low intensity signal on T1-weighted images and a high intensity signal on T2-weighted images (Figures 3A and B). In addition, the edge of the tumor was slightly enhanced by gadolinium, while the inside was heterogeneous (Figure 3C). Magnetic resonance cholangiopancreatography (MRCP) revealed local dilatation of IHBD. Based upon these examinations, one of the differential diagnoses was biloma at the cut end of the previous hepatectomy. To further investigate the content of the tumor, US-guided biopsy was performed. An aspirated sample contained a small volume of yellow and clear fluid, but bile was not found. Histological examination of the biopsy

specimens revealed a mucinous adenocarcinoma. Finally, the tumor was judged to be a recurrence of metastatic cancer with invasion to the IHBD. There was no evidence of metastasis in any other organs or in regional lymph nodes. The patient was considered to be a candidate for surgery. On March 13 in 2002, he underwent the third operation. When the peritoneal cavity was entered, there was no evidence of peritoneal dissemination, enlarged lymph nodes or ascites. The tumor was located in the liver in S8 adhering to the diaphragm and was about 4 cm in diameter, as measured by US. US was also performed to indicate a sufficient surgical margin of at least 15 mm. The tumor was resected using intermittent clamping of the primary branch of Glisson's triad (Pringle's procedure). When we cut the regional Glisson's triad, mucinous bile was found at the cut end of the bile duct. The portal vein was intact. According to the preoperative informed consent, partial resection of the liver in sub-segment 8 and the diaphragm was performed. Histological examination of the resected specimens revealed a well-differentiated mucinous adenocarcinoma, which was consistent with the metastatic lesion from colon cancer. The tumor extended along the lumen of the biliary ducts, replacing the non-neoplastic epithelium (Figure 4). The peripheral bile ducts were obstructed by the cancer cells, and the cut end of the bile duct was positive for cancer. The tumor invaded only the abdominal side of the diaphragm and was not apparent on the thoracic side. Immunohistochemically, the tumor

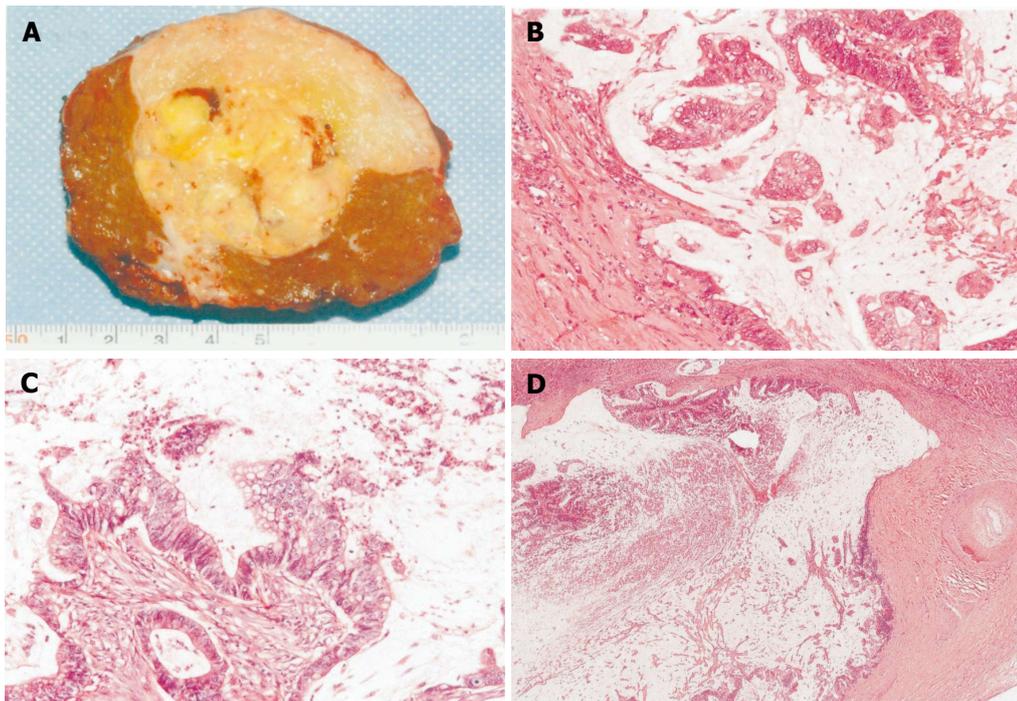


Figure 4 Macroscopic finding (A), well-differentiated mucinous adenocarcinoma (B) similar to cecal cancer (C), extension of tumor cells along the lumen of the biliary ducts with the non-neoplastic epithelium replaced (D). (B) HE, $\times 100$; (C) HE, $\times 100$; (D) HE, $\times 18$.

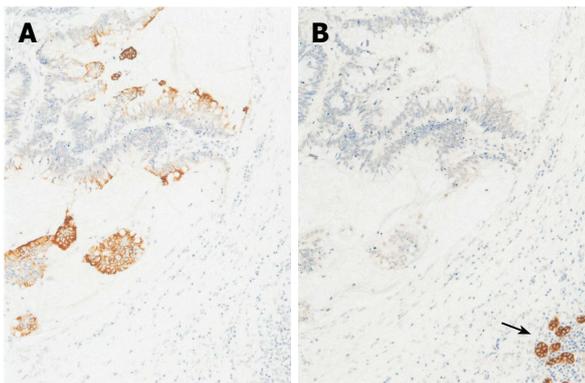


Figure 5 Immunohistochemically, tumor cells positive for CK 20 (A), but negative for CK7 (B), normal epithelial cells of IHBD positive for CK7 (arrow). (A) $\times 100$; (B) $\times 100$.

cells were positive for CK 20, but negative for CK7 (Figure 5). Thus, we diagnosed it as a recurrence of metastatic adenocarcinoma from cecal cancer.

Three months after the hepatectomy, the patient exhibited a recurrence at the cut surface of the residual liver. He and his family did not hope further surgery. Although he received chemotherapy using 5-FU for the local recurrence, he died 2 years and 9 mo after the second hepatectomy.

DISCUSSION

Liver metastases occur in 25%-35% of patients with colorectal cancer^[1]. Surgical resection has become a recognized curative treatment^[1,5,6]. However, the rate of intrahepatic recurrence has been reported to range from 16% to 28%^[5,7], and 9%-25% of these arise at the surgical margin^[8,9]. Okano *et al*^[3] reported that 42% of patients who have undergone hepatectomy for colorectal

liver metastases exhibit bile duct invasion, 12% of which exhibit macroscopic invasion. Among these metastases, an intrabiliary extension pattern is rare. Kubo *et al*^[4] reported that 3.7% of resected colorectal liver metastases are extended predominantly along the bile duct without forming an extrabiliary mass. The prognosis of patients with macroscopic bile duct invasion is better than that of patients with microscopic intraluminal invasion, which tends to aggressively involve the Glisson's triad^[3,4].

In the present case, we initially considered the cystic lesion to be a post-operative collection of inflammatory fluid. However, the lesion turned out to be a cystic tumor including mucin produced by the remnant cancer cells. Cancer cells and mucin obstructing the IHBD, caused dilatation of the peripheral bile ducts. Microscopically, about 10% of metastatic tumors from colorectal carcinoma exhibit mucinous features^[2]. Furthermore, the frequencies of incidence of localized IHBD dilatation caused by metastatic liver cancer and cystic degeneration of metastatic tumor have been reported to be 6%^[10] and 2%-4%, respectively^[11,12]. Therefore, the present case was considered to be a very rare case and preoperative diagnosis could not be confirmed by imaging examination alone. On the other hand, it is known that cholangiocellular carcinoma (CCC) is often accompanied with dilatation of the IHBD, because intrahepatic CCC exhibits bile duct extension, either through infiltration into the periductal tissues or appearing as a cast-like growth into the ductal lumen^[13]. Furthermore, CCC sometimes exhibits mucin production. Thus, the growth of colorectal metastases with bile duct invasion can be indistinguishable from that of less aggressive CCC, and it is sometimes difficult to make a differential diagnosis histologically. To discriminate liver metastases from primary CCC, immunostaining for CK7 and CK20 can be very useful. A CK20-positive and CK7-negative pattern is highly characteristic of liver metastases from colorectal cancer, compared with most

adenocarcinomas including CCC that are usually CK20-negative and CK7-positive^[14-16]. In the present case, this immunohistochemical finding allowed us to make a definite diagnosis.

Our patient has survived for more than two-years, in spite of local recurrence. This may be a consequence of the less aggressive features of this tumor, which exhibits macroscopic bile duct extension^[3,4]. However, because this invasion pattern tends to make the cut ends positive for cancer cells, it appears that it is necessary to examine the cut end of Glisson's triad by stamp cytology or frozen section pathology. Actually, during the 2nd hepatectomy, mucinous bile was found in the bile duct. However, since the tumor was a metastatic colorectal cancer rather than a CCC, partial hepatectomy was a procedure of choice for us. For colorectal metastases, we usually perform partial hepatectomy with a sufficient margin rather than anatomical resection if possible. Recently, Pawlik *et al*^[9] reported that the width of a negative surgical margin does not affect survival, recurrence risk, or site of recurrence. However, after having experienced our present case, anatomical hepatic resection may be a treatment of choice for metastatic liver tumor with bile duct extension to avoid the potential for a positive surgical margin. Further studies are necessary to develop a suitable surgical procedure for this unusual pattern of liver metastasis.

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CASE REPORT

Obstructive jaundice caused by secondary pancreatic tumor from malignant solitary fibrous tumor of pleura: A case report

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(α -SMA) or CD117, but positive for vimentin, CD34 and CD99. These findings are consistent with those on malignant solitary fibrous tumor of the pleura. We report the first case of obstructive jaundice caused by a secondary pancreatic tumor from malignant solitary fibrous tumor of the pleura.

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Key words: Malignant solitary fibrous tumor of the pleura; Secondary pancreatic tumor; Obstructive jaundice

Yamada N, Okuse C, Nomoto M, Orita M, Katakura Y, Ishii T, Shinmyo T, Osada H, Maeda I, Yotsuyanagi H, Suzuki M, Itoh F. Obstructive jaundice caused by secondary pancreatic tumor from malignant solitary fibrous tumor of pleura: A case report. *World J Gastroenterol* 2006; 12(30): 4922-4926

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Abstract

A 77-year-old man on systemic chemotherapy against postoperative bilateral multiple lung metastases of malignant solitary fibrous tumor of the pleura suffered from pruritus and jaundice. Blood examination showed elevated levels of hepatobiliary enzymes. Abdominal computed tomography showed a tumor with peripheral enhancement in the pancreatic head, accompanied with the dilatation of intra- and extra-hepatic bile ducts. He was diagnosed as having obstructive jaundice caused by a pancreatic head tumor. The pancreatic head tumor was presumably diagnosed as the metastasis of malignant solitary fibrous tumor of the pleura, because the findings on the pancreatic head tumor on abdominal CT were similar to those on the primary lung lesion of malignant solitary fibrous tumor of the pleura. The pancreatic tumor grew rapidly after the implantation of metallic stent in the inferior part of the common bile duct. The patient died of lymphangitis carcinomatosa of the lungs. Autopsy revealed a tumor that spread from the pancreatic head to the hepatic hilum. Microscopically, spindle-shaped cells exhibiting nuclear atypicality or division together with collagen deposition were observed. Immunohistochemically the pancreatic head tumor cells were negative for staining of α -smooth muscle actin

INTRODUCTION

Solitary fibrous tumor of the pleura (SFTP) is a neoplasm derived from mesenchymal cells located in the submesothelial lining of the pleural space, predominantly composed of spindle-shaped cells in combination with collagen deposition^[1]. SFTP comprises approximately 5% of primary pleural tumors following malignant mesothelioma^[2]. Seven percent to 13% of SFTPs are considered to be malignant neoplasms, of which 41% to 63% recur in the pleura or lung or metastasize to the liver, brain, spleen, adrenal gland and other organs^[1,2]. However, no metastasis to the pancreatic head with obstructive jaundice has been reported. Here, we report the first case of obstructive jaundice caused by a secondary pancreatic tumor from malignant SFTP.

CASE REPORT

The patient was a 77-year-old Japanese man. In July 2001, he was found to have an extrapleural tumor projecting from the right diaphragm by mass screening chest roentgenography. Partial resections of the right diaphragm and lower right lobe of the lungs were carried out in July 2002,



Figure 1 An extrapleural tumor projecting from the right diaphragm observed on chest roentgenography.



Figure 3 Multiple nodular lesions in the bilateral lung fields observed on chest computed tomography (CT).

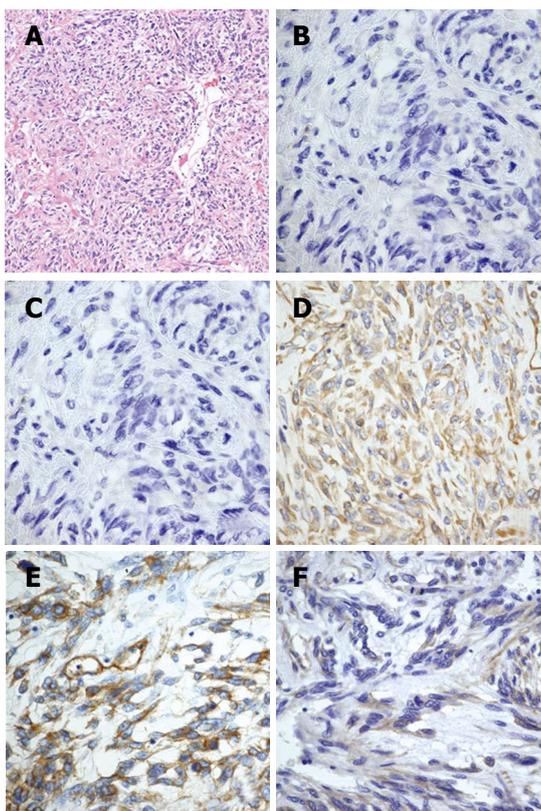


Figure 2 Spindle-shaped cells exhibiting nuclear atypicality or division together with collagen deposition (hematoxylin and eosin staining) (A), tumor cells negative for SMA (B) or CD117 (c-kit) (C) and positive for vimentin (D), CD34 (E) and CD99 (F) on histological examination.

because of the rapid tumor growth (Figure 1). The tumor in the resected specimens was composed of nonorganized, spindle-shaped cells exhibiting nuclear atypicality or division together with collagen deposition. Immunohistochemical staining showed that the tumor cells were negative for α -SMA or CD117 (c-Kit) but positive for vimentin, CD34 and CD99 (Figures 2A-F). He was diagnosed as having a solitary fibrous tumor of the pleura (SFTP).

In September 2003, multiple metastases were found in the bilateral lung fields and he received systemic chemotherapy with gemcitabine (GEM) and cisplatin (CDDP). Two courses of chemotherapy with GEM and CDDP temporarily reduced the tumor size in the lung.

In mid-April 2004, he experienced pruritus and showed

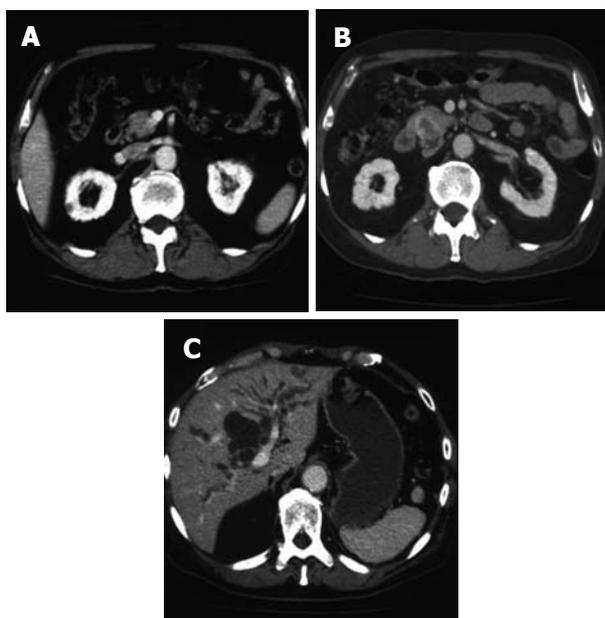


Figure 4 The pancreatic head tumor not detected by preoperative abdominal CT (A), a 25 mm \times 17 mm tumor in the pancreatic head detected by abdominal CT with its periphery enhanced by contrast material in the delayed phase (B) and dilatation of intra- and extra-hepatic bile ducts observed (C).

skin jaundice. He was found to have liver dysfunction. He was admitted to our hospital for further evaluation and treatment.

His medical history before 2001 was uneventful. He had a history of daily alcohol intake for 50 years and daily smoking for 40 years.

On the day of admission, he was alert. His body temperature was 35.4°C. His bulbar conjunctiva and skin were icteric. There was a surgical scar on his right lateral thorax. His liver was soft and palpable 3 cm below the umbilical margin. No tumor was palpated in the abdomen. A moveable and elastic tumor 2 cm in diameter was palpable in his right buttock.

The levels of hepatobiliary enzymes and bilirubin were elevated. The levels of tumor markers including carcino-embryonic antigen (CEA) and pancreatic cancer-associated antigen-2 (DUPAN-2) were normal (Table 1). Multiple

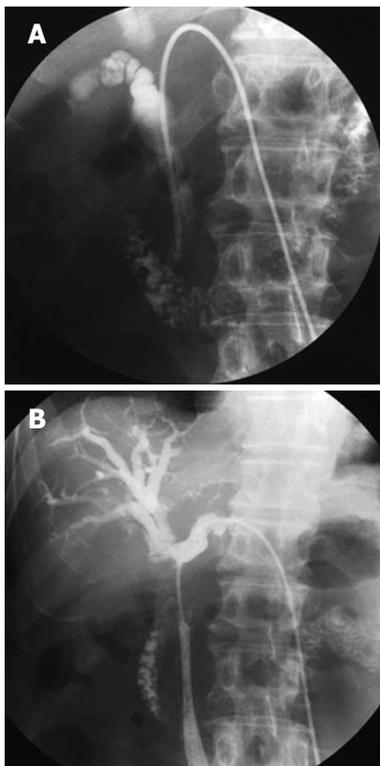


Figure 5 A marked stenosis of the inferior part of the CBD observed on cholangiography via a PTBD tube (A), an obstruction from inferior part of CBD to the confluence of the right and left extrahepatic bile ducts involving the whole stent on reperfomed cholangiography (B) on microscopic examination.

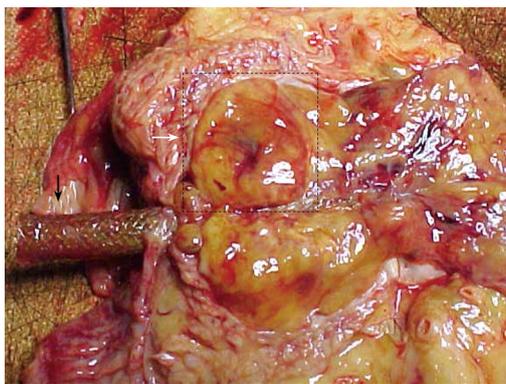


Figure 6 Macroscopic findings on autopsy. A dissected (120 mm × 30 mm × 20 mm) tumor spreading from the pancreatic head to the hepatic hilum and involving bilateral hepatic ducts. White arrow shows the pancreatic head tumor and black arrow shows the metallic stent.

nodular lesions were observed in the bilateral lung fields by chest computed tomography (CT) (Figure 3). By abdominal CT, a tumor (25 mm × 17 mm) with its periphery enhanced by the contrast material in the delayed phase, was observed in the pancreatic head (Figure 4B). This tumor was not detected by preoperative abdominal CT in 2002 (Figure 4A). The dilatation of intra- and extra-hepatic bile ducts was also observed. Intra-abdominal lymph nodes were not swollen (Figure 4C). Because the radiological findings observed with or without contrast material were similar to those observed in the primary pleural tumor, he was diagnosed as having obstructive jaundice presumably caused by a secondary pancreatic head tumor from malignant SFTP. Percutaneous transhepatic bile duct drainage (PTBD) was performed to improve his jaundice. Cholangi-

Table 1 Laboratory findings on admission

Peripheral blood		TP	6.3 mg/dL
WBC	4900 /μL	Alb	3.8 mg/dL
Hb	13.8 g/dL	Cr	0.96 mg/dL
Hct	41.2%	BUN	13.4 mg/dL
Plt	16.5 × 10 ⁴ μL	Amy	107 IU/L
Coagulation		FBS	111 mg/dL
PT	100%	CRP	0.08 mg/dL
APTT	28.5 (cont: 29.3)	Tumor Markers	
Fib	499 mg/dL	CA 19-9	16 U/mL
Blood Chemistry		CEA	4.2 ng/mL
T-Bil	4.2 mg/dL	DUPAN-2	135 U/mL
D-Bil	3.4 mg/dL	SCC	1.2 ng/mL
AST	318 IU/L	NSE	6.6 ng/mL
ALT	444 IU/L		
LDH	405 IU/L		
ALP	1860 IU/L		
γ-GTP	612 IU/l		

ography via a PTBD tube showed marked stenosis of the inferior part of the common bile duct (CBD) (Figure 5A). No atypical cell was observed in the bile collected by the tube.

Because of the bilateral multiple lung and right-buttock tumors, which were considered to have metastasized from the pleura, pancreatoduodenectomy was no longer an indication for treatment.

Thus, a metallic stent was implanted in the inferior part of CBD as a palliative treatment. The levels of hepatobiliary enzymes became normal after the implantation. However, in July 2004, he suffered from fever and jaundice with elevated levels of hepatobiliary enzymes. Abdominal CT demonstrated obstruction of the stent and dilataion of the intrahepatic bile ducts. Furthermore, an obstruction from the inferior part of CBD to the confluence of the right and left extra-hepatic bile ducts involving the whole stent was observed when cholangiography was performed again (Figure 5B). Although a drainage tube was reinserted in the anterior and lateral branches of the intra-hepatic bile ducts, he developed lymphangitis carcinomatosa and died of respiratory failure.

Autopsy was performed with informed consent. Multiple tumors were observed in the bilateral lung. A solitary tumor (25 mm × 15 mm × 15 mm) was observed in his right buttock. A large tumor (120 mm × 30 mm × 20 mm) spread from the pancreatic head to the hepatic hilum and involved bilateral hepatic ducts (Figure 6).

Microscopically, spindle-shaped cells exhibiting nuclear atypicality or division together with collagen deposition were observed (Figure 7A). Immunohistochemically, tumor cells of the pancreatic head were negative for α-SMA or CD117, but positive for vimentin, CD34 and CD99 (Figures 7B-F). These findings were consistent with those on malignant SFTP resected in July 2002.

DISCUSSION

Malignant SFTP recurs in the pleura or lung or metasta-

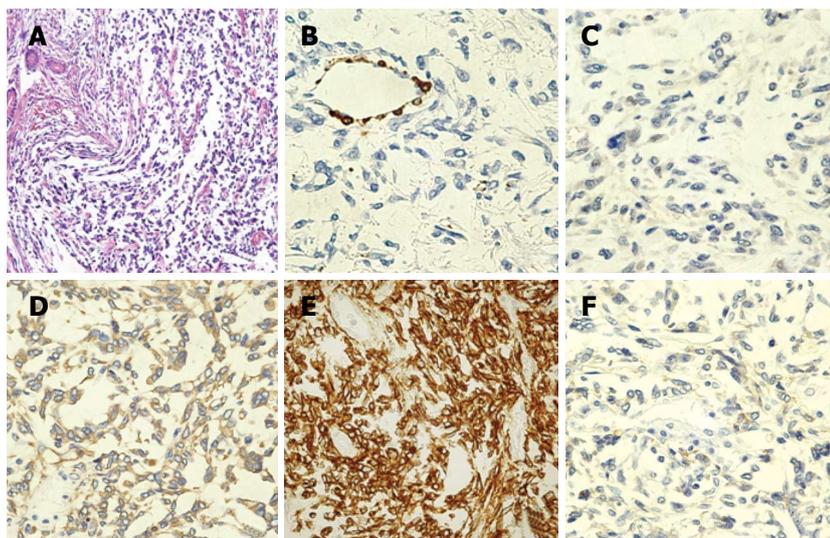


Figure 7 Spindle-shaped cells exhibiting nuclear atypicity or division together with collagen deposition (hematoxylin and eosin staining) (A), tumor cells negative for SMA (B) or CD117 (c-kit) (C) and positive for vimentin (D), CD34 (E) and CD99 (F) on histological examination. These findings are consistent with those on malignant solitary fibrous tumor of the pleura, resected in July 2002.

sizes to the liver, brain, spleen, adrenal gland and other organs *via* a blood-borne pathway^[1-3]. This present patient was considered to have a pancreatic head tumor having metastasized from a malignant SFTP for the following reasons. Multiple metastatic lesions in the bilateral lung and a growing subcutaneous tumor were observed and the findings on the pancreatic head tumor by abdominal CT were similar to those on the primary lung SFTP. The tentative diagnosis was confirmed by the histopathological and immunohistochemical findings on autopsy.

Secondary pancreatic tumor is rare. Nakamura *et al*^[4] reported that the incidence of secondary pancreatic tumor is approximately 6% in autopsy studies and increases to approximately 15% in cases of malignant tumors. The lung is the second most common primary site (18%) of secondary pancreatic tumor^[4,5]. The histopathological types of this tumor are large cell carcinoma, small cell carcinoma, adenocarcinoma and squamous cell carcinoma^[4-7]. Malignant SFTP has never been reported as a cause of secondary pancreatic tumor to date.

In general, secondary pancreatic tumor is caused by blood-borne metastasis, lymphatic metastasis and tumor infiltration from adjacent organs or peritoneal seeding. The secondary pancreatic tumor of this present patient was considered to be caused by blood-borne metastasis because no metastasis in regional lymph nodes was observed during operation or on autopsy, but multiple metastatic lesions in the bilateral lungs and a subcutaneous metastatic tumor in the right buttock were observed.

Although radiation therapy and chemotherapy have been performed against malignant SFTP in addition to resection, they could not achieve sufficient therapeutic efficacy^[2]. On the other hand, GEM monotherapy or combination therapy with CDDP is effective against malignant mesothelioma^[8].

Chemotherapy with GEM and CDDP, which is effective for patients with malignant mesothelioma, was selected for this patient because the treatment of malignant SFTP with metastasis was not authorized, and both malignant mesothelioma and SFTP originated from the pleura. However, the tumor grew more rapidly even after chemother-

apy. Forty-one percent to 63% of malignant SFTPs recur locally or metastasize to other organs^[1,2] and 23% of these patients die within 24 mo of diagnosis^[9]. Therefore, the establishment of an effective anti-tumor therapy against malignant SFTP is desired.

The leading drug for the novel therapy is imatinib mesylate, which is effective against gastrointestinal stromal tumors (GISTs)^[10]. In half of the patients with malignant SFTP, tumor cells have been found positive for c-kit^[11]. Therefore, imatinib mesylate, which inhibits kit-tyrosine kinase, may be a promising drug for molecular-target-based therapy. This hypothesis should be verified in future studies.

In summary, we report a rare case of obstructive jaundice caused by a secondary pancreatic tumor from malignant SFTP. Pancreatic metastasis may adversely affect the prognosis of malignant SFTP. Therefore, abdominal images and the levels of hepatobiliary enzymes should be examined regularly.

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Pancreatitis complicating mucin-hypersecreting common bile duct adenoma

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Abstract

Villous adenomas of the bile ducts are extremely uncommon. We describe a 58-year-old man presenting with clinical signs and laboratory findings of acute pancreatitis and obstructive jaundice. Preoperative investigation demonstrated a dilated papillary orifice with mucus exiting (fish-mouth sign) and a filling defect in the distal common bile duct. He underwent a modified Whipple operation and histological examination of the surgical specimen showed villous adenoma with rich secretion of mucus.

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Key words: Villous adenoma; Common bile duct; Endoscopic retrograde cholangiopancreatography

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INTRODUCTION

Villous adenomas are benign epithelial lesions with malignant potential, which are usually encountered in the colon, less commonly in the small bowel or the ampulla of Vater, and extremely uncommonly in the bile ducts^[1]. Since Saxe *et al* first reported a case of villous adenoma of the

common bile duct (CBD) in 1988, a total of 17 cases have been reported so far in the literature^[1-17].

We present what is to our knowledge, the first case of a mucin-secreting CBD adenoma presenting as acute pancreatitis due to mucus hypersecretion and showing the endoscopic finding of "fish-mouth" in the papilla of Vater.

CASE REPORT

A 58-year-old man was admitted to the department of Internal Medicine complaining for upper abdominal pain radiating to the back, nausea and vomiting during the last six hours.

Physical examination demonstrated a well nourished man who appeared jaundiced. The abdomen was soft but revealed tenderness in the epigastrium and a movable, firm, non-tender mass in the right upper quadrant, which was felt to be a distended gallbladder. Previous medical history included a 3-mo period of dyspepsia. The patient did not mention previous episodes of fever or weight loss. He had never been abroad, was not on any drug or alcohol abuse and had no history of serious illness.

Laboratory investigations showed a total serum bilirubin level of 51 $\mu\text{mol/L}$ (normal range 0-3 $\mu\text{mol/L}$), direct bilirubin level of 39 $\mu\text{mol/L}$, alkaline phosphatase level of 548 U/L (normal range < 120 U/L), alanine transaminase level of 143 U/L (normal range < 45 U/L) and serum amylase level of 1720 U/L (normal range < 30 U/L). The hemogram revealed hemoglobin and hematocrit of 142 g/L and 44.6%, respectively, while white blood cell count was 16 100 /mm³. On abdominal ultrasound and CT the pancreas was edematous, the gallbladder was distended without calculi and the CBD was found to be dilated (12.5 mm), with a nonshadowing tissue mass in its distal end. The patient was treated conservatively with resolution of symptoms.

On seventh day, an endoscopic retrograde cholangiopancreatography (ERCP) was performed and demonstrated a patulous papillary orifice of the major papilla (fish-mouth sign) with mucus exuding from it (Figure 1), a normal pancreatogram and a 2 cm \times 1 cm intraluminal filling defect in the distal CBD (Figure 2).

A sphincterotomy was performed and in order to rule out the presence of stone, a balloon was inserted, but nothing was retrieved. A subsequent lithotripter-basket extraction took a fragment tissue out and a 8.5-French Cotton-Leung type plastic stent of 7 cm was placed.

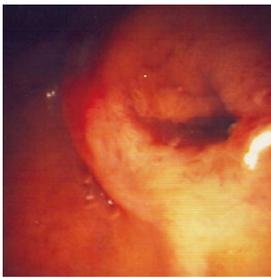


Figure 1 Endoscopic view showing a dilated papillary orifice exiting mucus.



Figure 2 ERCP demonstrating an intraluminal filling defect in the distal common bile duct. A 8.5Fr stent has been placed.

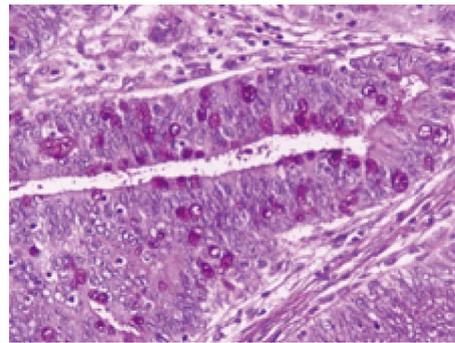


Figure 3 Surgical specimen showing that the tumor consists of tall, columnar, pseudostratified epithelial cells with elongated or round atypical nuclei. Some of the cells contain large amounts of mucin within the apical cytoplasm (PAS X 400).

Because the histological examination of the retrieved tissue showed that the main mass was a villous adenoma, the mass was thought to be resectable with safety margins. The patient subsequently underwent a modified Whipple procedure. The histopathological examination of the specimen demonstrated that the tumor consisted of finger-like villous or papillary processes containing central thin cords of lamina propria lined by a neoplastic epithelium. The epithelial cells were tall columnar, pseudostratified with elongated or round atypical nuclei. Some of the cells contained large amounts of mucin within the apical cytoplasm. Focally goblet cells lied within the epithelium (Figure 3). No signs of hyperplasia or adenoma of the pancreas were observed in the specimen.

The patient recovered uneventfully and remains in good general condition, six months after discharge.

DISCUSSION

Adenomas of the bile ducts are divided into papillary adenomas, pedunculated adenomas and sessile adenomas, according to the gross configuration of the tumors^[18]. This classification replaced an earlier classification of papillomas and adenomas. Villous adenomas are thus classified as frond-like sessile adenomas^[3]. Histologically, however, adenomas are classified into tubular, tubulovillous and villous adenomas.

The most common site for villous adenomas in the biliary tree is the CBD. These adenomas usually develop in the distal aspect of the CBD^[18]; they are histologically similar to villous adenomas in the ampullary region, gallbladder and intestine and should probably be considered having similar biological behavior. The adenoma to carcinoma sequence is well accepted in the colon and most likely also applies in the ampullary region, gallbladder and bile ducts^[19].

Of note, in reviewing the reported cases^[1-17] of bile duct villous adenomas, the clinical picture includes painless jaundice, pruritus, upper abdominal pain, cholangitis and dyspepsia. The tumors rarely grow large enough to become palpable because of their site. The degree of jaundice has been described to fluctuate in some reports, which may be attributable to a ball valve effect of the tumor. Preoperative diagnosis or diagnosis without operation was possible in 6 out of 17 cases, mainly in recently reported cases, reflecting the current sophisticated diagnostic and therapeutic techniques of the biliary tract and pancreas. Abdominal US and CT demonstrated dilation of the CBD and an intraductal tumor but were not capable of specifying any further the nature of the lesion. The absence of gallbladder calculi, as well as the absence of acoustic shadowing of the CBD mass in the patients, made the possibility of the lesion being calculus unlikely. In addition, in the absence of gallbladder sludge, sludge in the CBD is unusual. Moreover, differentiation of a villous adenoma from other nonshadowing solid lesions, such as blood clots, nonshadowing calculi, lipomas, fibromas, or carcinoid tumors on the basis of US and CT findings is difficult^[5]. Echoendoscopy is a well proven technique with higher image resolution and greater diagnostic sensitivity than conventional imaging methods and would be useful to identify the nature of the lesion and exclude an invasive component.

It must be emphasized that two cases of coexisting biliary tract and intestinal polyps have been reported^[20,21]. It has been proposed that bile duct adenomas are part of the spectrum of generalized gastrointestinal polyposis. Some authors^[20-23] have suggested that upper gastrointestinal surveillance of patients with familiar adenomatous polyposis should routinely include imaging of the biliary tract.

Our case is both unique and challenging in that the CBD adenoma had close resemblance to intraductal papillary mucinous tumors (IPMT) of the pancreas. More specifically, it was composed of secreting cells, with hypersecretion of mucin. The hypersecreted viscous mucin led to obstruction of the papillary orifice, thus increasing the pancreatic intraductal pressure and activating the cascade of mechanism of pancreatitis. Moreover, the "fish-mouth" sign in the major papilla is an endoscopic finding

which has been discussed in mucin-secreting pancreatic tumors. As both lesions are encountered premalignant, complete resection of both of them is considered mandatory. Therefore, we suggest that the term IPMT of the bile ducts could be used to describe this rare condition.

In conclusion, our case reflects the need to consider a wide differential in patients presenting with acute pancreatitis and jaundice, especially in the absence of cholelithiasis and alcohol abuse.

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LETTERS TO THE EDITOR

Therapeutic endoscopic retrograde cholangiopancreatography and related modalities have many roles in hepatobiliary hydatid disease

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Abstract

The authors report their experience about 8 cases of intrabiliary rupture of hepatobiliary hydatid disease, and add an algorithm for treatment. To our opinion, the use of diagnostic and therapeutic endoscopic retrograde cholangiopancreatography (ERCP) in the management of hepatobiliary hydatid disease was not stated properly in their proposed algorithm. According to the algorithm, the use of ERCP and related modalities was only stated in the case of postoperative biliary fistulae. We think that postoperative persistent fistula is not a sole indication, there are many indications for ERCP and related techniques namely sphincterotomy, extraction, nasobiliary drainage and stenting, in the treatment algorithm before or after surgery.

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Key words: Therapeutic endoscopic retrograde cholangiopancreatography; Hepatobiliary; Hydatid

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TO THE EDITOR

We have read with great interest the article titled "Intrabiliary rupture: an algorithm in the treatment of controversial complication of hepatic hydatidosis"^[1]. The authors reported their experience about 8 cases of intrabiliary rupture of hepatobiliary hydatid disease, and added an algorithm for treatment. To our opinion, the

use of diagnostic and therapeutic endoscopic retrograde cholangiopancreatography (ERCP) in the management of hepatobiliary hydatid disease was not stated properly in their proposed algorithm. According to the algorithm, in a case of preoperative suspicion of intrabiliary rupture due to various reasons, such as cystic content in common bile duct (CBD), dilated CBD and obstructive jaundice, surgery was proposed without prior to ERCP. The use of ERCP and related modalities was only stated in the case of postoperative biliary fistula. Many reports^[2-4] including ours^[5,6] are published in English literature about the use of ERCP in the management of hydatid disease. Our former report^[5] has reviewed a total of 294 cases, after collecting 273 cases in 26 articles and 6 abstracts from literature and adding 21 cases of our own experience. Considering the current literature^[7,8] and our experience, we think that postoperative persistent fistula is not a sole indication, there are many indications for ERCP and related techniques namely sphincterotomy, extraction, nasobiliary drainage and stenting, in the treatment algorithm before or after surgery.

ERCP in the preoperative period^[2] I - defines the cystobiliary relationship to help in surgery planning, II - permits evaluation of acute conditions like cholangitis and obstruction so that subsequent surgery can be performed on an elective basis, III - may give permanent cure specifically in cases of frank intrabiliary rupture if evacuation of biliary tract and cystic cavity is manageable, and IV - when combined with preoperative endoscopic sphincterotomy may decrease the incidence of the development of postoperative external fistulae. While the first three statements have been studied extensively, the fourth statement may warrant further studies to clarify the criteria of selection of appropriate cases. The only study regarding this issue performed by Galati *et al*^[7], reported a significant decrease in postoperative fistulae in cases that underwent selective preoperative ERCP (3.8% *versus* 7.4%).

ERCP in the postoperative period^[2] I - can help to clarify the causes of ongoing or recurrent symptoms or laboratory abnormalities, II - may help to resolve the obstruction or cholangitis due to residual material in biliary ducts, III - may provide the chance to manage postoperative external biliary fistulae, and IV - may be a realistic solution for secondary biliary strictures^[8].

Since hydatid disease is a serious public health problem despite the use of various kinds of preventive measures,

we greatly appreciate every kind of studies regarding the issue to solve the controversions. Endoscopic therapy should be incorporated into the other treatment options including surgery, percutaneous measures and chemotherapy with benzimidazole compounds. The exact place of each therapeutic modality in a particular case should be decided on the basis of expanding current literature.

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Xin-Xin Zhang, Professor

Department of Infectious Disease, Rui Jin Hospital, 197, Rui Jin Er Road, Shanghai 200025, China

Meetings

MAJOR MEETINGS COMING UP

First Biennial Congress of the Asian-Pacific Hepato-Pancreato-Biliary Association
March, 2007
Fukuoka, Japan
<http://www.congre.co.jp/1st-aphba>

American College of Gastroenterology
Annual Scientific
20-25 October 2006
Las Vegas, NV

14th United European Gastroenterology
Week, UEGW
21-25 October 2006
Berlin, Germany

APDW 2006: Asian Pacific Digestive Week
2006
26-29 November 2006
Lahug Cebu City, Philippines

EVENTS AND MEETINGS IN THE UPCOMING 6 MONTHS

Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
24-25 March 2006
Sydney - NSW
Falk Foundation e.V.
symposia@falkfoundation.de

10th International Congress of Obesity
3-8 September 2006
Sydney
Event Planners Australia
enquiries@ico2006.com
www.ico2006.com

Easl 2006 - the 41st annual
26-30 April 2006
Vienna, Austria
Kenes International

Prague hepatology 2006
14-16 September 2006
Prague
Foundation of the Czech Society of Hepatology
veronika.revicka@congressprague.cz
www.czech-hepatology.cz/phm2006

12th International Symposium on Viral Hepatitis and Liver Disease
1-5 July 2006
Paris
MCI France
isvhld2006@mci-group.com
www.isvhld2006.com

Falk Symposium 152: Intestinal Disease Part I, Endoscopy 2006 - Update and Live Demonstration
4-5 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

Falk Symposium 153: Intestinal Disease Part II, Immunoregulation in Inflammatory Bowel Disease - Current Understanding and Innovation
6-7 May 2006
Berlin
Falk Foundation e.V.
symposia@falkfoundation.de

ILTS 12th Annual International Congress
3-6 May 2006
Milan
ILTS
www.its.org

Internal Medicine: Gastroenterology
22 July 2006-1 August 2006
Amsterdam
Continuing Education Inc
jbarnhart@continuingeducation.net
6th Annual Gastroenterology And

Hepatology
15-18 March 2006
Rio Grande
Office of Continuing Medical Education
cmenet@jhmi.edu
www.hopkinscme.net

World Congress on Gastrointestinal Cancer
28 June 2006-1 July 2006
Barcelona, Spain
c.chase@imedex.com

International Conference on Surgical Infections, ICSI2006
6-8 September 2006
Stockholm
European Society of Clinical Microbiology and Infectious Diseases
icsi2006@stocon.se
www.icsi2006.se/9/23312.asp

7th World Congress of the International Hepato-Pancreato-Biliary Association
3-7 September 2006
Edinburgh
Edinburgh Convention Bureau
convention@edinburgh.org
www.edinburgh.org/conference

Society of American Gastrointestinal Endoscopic Surgeons
26-29 April 2006
Dallas - TX
www.sages.org

Digestive Disease Week 2006
20-25 May 2006
Los Angeles
www.ddw.org

Annual Postgraduate Course
25-26 May 2006
Los Angeles, CA
American Society of Gastrointestinal Endoscopy
www.asge.org/education

American Society of Colon and Rectal Surgeons
3-7 June 2006
Seattle - Washington
www.fascrs.org

EVENTS AND MEETINGS IN 2006

10th World Congress of the International Society for Diseases of the Esophagus
22-25 February 2006
Adelaide
isde@sapmea.asn.au
www.isde.net

Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
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www.cag-acg.org

Canadian Digestive Disease Week
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Banff, Alberta
Digestive Disease Week Administration
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Prague Hepatology 2006
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www.czech-hepatology.cz/phm2006

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MCI France
isvhld2006@mci-group.com
www.isvhld2006.com/

Falk Seminar: XI Gastroenterology Seminar Week
4-8 February 2006
Titisee
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symposia@falkfoundation.de

European Multidisciplinary Colorectal Cancer Congress 2006
12-14 February 2006
Berlin
Congresscare
info@congresscare.com
www.colorectal2006.org

Falk Symposium 152: Intestinal Disease Part I, Endoscopy 2006 - Update and Live Demonstration
4-5 May 2006
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Falk Symposium 153: Intestinal Disease Part II, Immunoregulation in Inflammatory Bowel Disease - Current Understanding and Innovation
6-7 May 2006
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United European Gastroenterology Federation
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ILTS 12th Annual International Congress
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XXX Panamerican Congress of Gastroenterology
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Cancun
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EVENTS AND MEETINGS IN 2007

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- 2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaobua Zazhi* 1999; **7**: 285-287

In press

- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci U S A* 2006; In press

Organization as author

- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462]

Both personal authors and an organization as author

- 5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764]

No author given

- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303]

Volume with supplement

- 7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325]

Issue with no volume

- 8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; **(401)**: 230-238 [PMID: 12151900]

No volume or issue

- 9 Outreach: bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

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- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

- 12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wiczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

- 13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ Cell Tumour Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

- 14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/EID/eid.htm>

Patent (list all authors)

- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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Post-transcriptional regulation of vascular endothelial growth factor: Implications for tumor angiogenesis

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Abstract

Vascular endothelial growth factor (VEGF) is a potent secreted mitogen critical for physiologic and tumor angiogenesis. Regulation of VEGF occurs at several levels, including transcription, mRNA stabilization, translation, and differential cellular localization of various isoforms. Recent advances in our understanding of post-transcriptional regulation of VEGF include identification of the stabilizing mRNA binding protein, HuR, and the discovery of internal ribosomal entry sites in the 5'UTR of the VEGF mRNA. Monoclonal anti-VEGF antibody was recently approved for use in humans, but suffers from the need for high systemic doses. RNA interference (RNAi) technology is being used *in vitro* and in animal models with promising results. Here, we review the literature on post-transcriptional regulation of VEGF and describe recent progress in targeting these mechanisms for therapeutic benefit.

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Key words: Vascular endothelial growth factor; Vascular endothelial growth hormone; Post-transcriptional regulation; mRNA stability; HuR; ELAV1; Internal ribosomal entry; IRES; siRNA; RNAi; Bevacizumab

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INTRODUCTION

The growth of any tissue *in vivo* relies on the adequacy of its vascular supply. Moreover, the notion of tumor

angiogenesis, first advanced by Folkman in the early 1970s, posits that the growth of any tumor beyond 1-2 cubic centimeters depends on the growth of new blood supply^[1]. In cases of insufficient vasculature, tissues become depleted of oxygen and nutrients, leading to the secretion of angiogenic factors^[2]. These factors, which include hormones, interleukins, insulin, and growth factors, spur the ingrowth of new blood vessels. Vascular endothelial growth factor (VEGF) is one such key regulator of angiogenesis. It is a secreted mitogen and the most potent angiogenic factor, specific to endothelial cells and highly expressed in areas of active angiogenesis such as solid tumors.

The upregulation of VEGF expression in response to hypoxia plays a crucial role in tumor angiogenesis. Though it is not itself an oncogene, VEGF is upregulated in tumorigenesis and is important in blood vessel formation in solid tumors^[3]. VEGF levels correlate with tumor progression and invasion, and a high VEGF level in colorectal carcinoma has been found to be an independent prognostic factor for long-term survival^[4,5].

Hypoxic induction of VEGF appears to occur both by transcriptional activation and through stabilization of VEGF mRNA, which is otherwise labile in normoxic conditions. Transcriptional activation of VEGF relies largely on binding to the hypoxia inducible factor-1 (HIF-1), a heterodimeric basic helix-loop-helix protein that activates transcription of the human erythropoietin gene in hypoxic cells^[6]. HIF-1 binds to a sequence in the 5'-flanking region of the VEGF gene called the hypoxic response element (HRE)^[7-9]. Several other molecules have been implicated in the transcriptional control of VEGF. *Sp1* stimulates transcription by binding to G/C-rich boxes present on the VEGF promoter^[10]. *AP-1* is a dimeric transcription factor of the leucine zipper family that is composed of jun/jun or jun/fos subunits. Hypoxia, oxidative stress, and cytokines may increase VEGF expression through the synthesis of jun and fos proteins and increased AP-1 binding activity. A number of other transcription factors also contribute to VEGF induction and regulation, including Stat-3^[11].

Alternative splicing

In humans there are five alternatively spliced isoforms of VEGF, each is named for the number of amino acids along its length (VEGF 206, -189, -165, -145, 121). While varying amounts of each VEGF isoform mRNA can be generated to produce certain or all isoforms of VEGF^[12],

each is predicted to have a characteristic extracellular localization based on biochemical differences. The larger isoforms bind neuropilin, matrix, and cell surface heparin proteoglycans, and are thought to act locally. The smaller isoforms do not display the heparin proteoglycan binding region and may diffuse to sites distant from the site of synthesis^[13,14].

Post-transcriptional Regulation

A body of evidence is growing rapidly to demonstrate that post-transcriptional regulation of VEGF is critically important in the fine-tuned response required for both physiologic and malignant expression. The importance of post-transcriptional regulation of VEGF is based upon several key observations about the intrinsic nature of the VEGF mRNA. First, the mRNA is labile under conditions of normal oxygen tension^[15]. Second, the 5' UTR of the VEGF mRNA possesses features that make efficient ribosome scanning and initiation of translation unwieldy.

Lability

Whereas the average half-life of eukaryotic mRNAs is 10-12 h, the half-life of VEGF mRNA is less than one hour^[16]. In 1995, Ikeda and colleagues first reported that although VEGF levels were elevated in cells cultured in hypoxic conditions, transcriptional activation alone could not account for the increase in VEGF mRNA levels. Nuclear run-on assays demonstrated upregulation of VEGF transcription under hypoxia that was apparent after three hours, and persisted after fifteen hours of incubation. VEGF mRNA levels were 8-10 times higher than baseline, and this persistent elevation of VEGF could only be explained by increased stability of the mRNA. They concluded that hypoxia could lengthen the half-life of VEGF mRNA by 2-3 fold^[7].

The effect of stabilization is mediated by the RNA-binding protein HuR. Steitz and colleagues first established the association between mRNA degradation and HuR, a member of the Elav family of proteins found in *Drosophila*^[17]. Beginning with the observation that some mRNAs are targeted for rapid degradation by the presence of AU-rich elements (AREs) in the 3' UTRs, it was determined that HuR exhibits affinity for AREs, and levels of HuR correlate with *in vitro* mRNA degradation^[18].

In VEGF, it was observed that the increase in mRNA stability coincided with the binding of a protein to an ARE, forming an RNA-protein complex in a hypoxia-inducible fashion^[16]. Using a tumor cell line lacking the wild type von Hippel-Lindau tumor suppressor gene and in which VEGF mRNA is constitutively stabilized, this RNA-protein complex was found to be constitutively elevated^[19]. The protein was later identified as the HuR protein^[15]. Inhibition of HuR expression by antisense sequences was found to inhibit the hypoxic stabilization of VEGF mRNA, demonstrating its critical role in post-transcriptional stabilization of VEGF expression. However, total cellular steady-state HuR was not altered by hypoxia, raising the possibility that HuR is a component of a hypoxia-inducible complex whose other components are regulated by oxygen tension^[15].

HuR probably has a function as a nuclear-cytoplasmic shuttle as well. It is predominantly localized to the nucleus, where it likely binds newly-transcribed VEGF mRNA and transports it to the cytoplasm and protects it from the ARE-mediated degradation pathways^[20].

Recent studies have suggested HuR and VEGF mRNA localize to the cytoplasm in response to cellular stress^[21]. Upon hypoxic stress, HuR is found to be localized to the cytosol and bound to target mRNAs, preventing their decay. New experiments using double labeling immunofluorescence show that VEGF and HuR colocalize to the nucleus during hypoxia^[22,23]. The nuclear role of VEGF mRNA, if any is unclear. Further studies to more clearly delineate the intracellular localization of VEGF mRNA and HuR are warranted.

The mechanism by which HuR protects VEGF from degradation has been further investigated. One hypothesis is that RNA binding proteins confer stability by binding directly to destabilizing sequences and making them unavailable to endonucleases. Goldberg and colleagues suggest that RNA-stabilizing factors may localize to a distinct binding site and thereby change the secondary or tertiary structure of the RNA, making a specific site unavailable to endonucleolysis. In support of this hypothesis, a 40 bp region adjacent to the HuR-binding site in the VEGF stability region has been identified that is susceptible to ribonucleases in the absence of HuR^[24].

HuR's importance in carcinogenesis is underlined by a report Lopez de Silanes and colleagues^[25]. Using a nude mouse model and the RKO colorectal cancer cell line, they reported that tumors modified to overexpress HuR grew significantly larger than controls, and conversely, tumors modified to repress HuR expression grew smaller. Whether this effect was brought about through stabilization of VEGF mRNA is yet unknown.

IRES

Internal ribosomal entry sites (IRESs) were first discovered in picornaviruses in which they initiate translation of naturally uncapped viral mRNA. Eukaryotic mRNA, however, possesses a 7-methylguanosine cap in the 5'UTR that is critical in the canonical model of translation. In this model, the eukaryotic initiation factors (eIFs) recognize and bind the 5' cap, and unwind the secondary structure of the 5'UTR, thereby making it sterically feasible for ribosomes to scan through the 5' UTR and translate the mRNA beginning at the AUG start codon^[26].

However, perhaps 3%-5% of all cellular mRNAs are translated by a mechanism independent of the 5' cap structure^[27]. Most of these mRNAs are likely to contain an internal ribosome-entry site (IRES) in the 5' untranslated region (UTR), as the ability to maintain efficient translation without utilizing the 5' cap-dependent mechanism relies on the presence of one or several IRESs. IRESs are typically found in mRNAs that possess unusually long 5'UTRs, greater than -300 nt, which consequently have significant secondary structure^[28]. In eukaryotes, IRES-mediated translation bypasses the canonical initiation step by directly recruiting ribosomes to the start codon without the need for the 5' mRNA cap structure or the eukaryotic initiation

factor (eIF) complex^[28,29].

The eIF-4F complex is one of the key mediators of ribosome recruitment in cap-dependent translation and the eIF-4E subunit of this complex can be rate-limiting^[2]. Under normal cellular conditions, eIF-4E is bound to its binding protein 4E-BP1 and translation is limited by the availability of the unbound fraction of eIF-4E^[30]. When 4E-BP1 is phosphorylated, eIF-4E is released and becomes available to bind mRNA and participate in the eIF-4F complex. This phosphorylation is instigated by the downstream effects of various mitogenic pathways. Hypoxia, however, has the opposite effect, increasing the binding of eIF-4E with 4E-BP1, which may mediate the global decrease in protein synthesis seen in stressed cells^[31].

Several observations about the 5' UTR of VEGF mRNA led to the hypothesis that it may contain IRES elements. First, cap-dependent translation of VEGF is cumbersome because the 5' UTR (1038 bp in humans) is much longer than typical eukaryotic 5' UTRs (-300 nt) and does not allow efficient ribosomal scanning. Second, it has a high G+C content, predisposing it to form stable secondary structures. Third, the 5'UTR contains a short open reading frame with in-frame initiation and termination codons^[32]. Furthermore, in order to maximize its effect, translation of VEGF must be upregulated during periods of stress such as cellular hypoxia, when cap-dependent protein synthesis is globally inhibited.

In 1998, VEGF mRNA was shown to possess two IRES sites (IRES A and B) in the 5' UTR^[32-34]. IRES A is contained in a 293nt segment just upstream from the AUG codon and is believed to control translation initiated at the AUG codon^[35]. IRES B is contained in the early portion of the 5' UTR^[33]. These two sites appear to bind different proteins, and the cellular milieu in which each functions optimally are not well understood.

IRES-mediated translation *in vivo* and *in vitro* yields a distinct isoform of VEGF, called "Large VEGF" or "L-VEGF." Whereas initiation mediated by the 5' cap and IRES A is at the AUG start codon it shares with cap-dependent translation, the initiation of the L-VEGF isoform occurs at an upstream CUG start codon (nt 499) within the 5'UTR. The L-VEGF isoform contains an N-terminal extension of 180 a.a., which is cleaved to yield a C-terminal peptide that is nearly identical to VEGF 189. This N-terminal extension is highly conserved through human, bovine, and murine cells, but whether it has any function is unknown^[36].

Inquiry into the control of IRES A has uncovered a complex model of translational regulation. The five classic isoforms (excluding L-VEGF) are the translation products of five distinct mature mRNAs; each of these mature mRNAs is the product of alternative splicing of the transcription product or pre-mRNA. Bornes and colleagues have reported that differences in exon content of the differentially spliced mRNA can lead to preferential activity of one IRES or the other^[35].

Though it is becoming clear that the IRES mechanism in VEGF translation is a plausible pathway in normal physiology, it remains debatable whether this pathway plays a significant role in tumor angiogenesis. The controversy relies on the observation that there is a correlation

between eIF-4E and protein synthesis in malignancy. This correlation has been demonstrated in breast carcinomas, head and neck squamous cell carcinomas, soft tissue sarcomas, and colon carcinoma^[37-40], and is bolstered by the observation that cells that over-express eIF-4E increase secretion of VEGF by greater than 100-fold^[41]. Therefore, it is conceivable that VEGF production in transformed cells relies on the cap-dependent pathway as well, though this has not been definitively demonstrated.

Whether hypoxia is a sufficient stimulus to induce IRES activity, or if other mitogenic stimuli can trigger internal ribosomal entry remains unknown. In a study comparing the IRES elements of VEGF, hypoglycemia was seen to significantly activate IRES activity^[42]. In both transformed and benign cells, the balance between IRES and cap-dependent translation in VEGF requires further investigation.

Deranged expression of the *c-myc* oncogene is associated with many human malignancies, as its over-expression promotes cell growth and angiogenesis^[43]. This effect is in part due to the fact that *myc* over-expression increases secretion of VEGF^[44]. The mechanism behind this increase was recently described by Mezquita and colleagues^[45]. They demonstrated that *myc* interacts with VEGF mRNA to upregulate initiation of translation capable of bringing about a 10-fold increase in VEGF production. They observed no increase in eIF activity and attribute the translational upregulation to VEGF-specific increase of translation initiation, either through *c-myc* or VEGF directly.

Implications for Tumor Angiogenesis

In 1993, Kim and colleagues reported that anti-VEGF antibodies had a strong inhibitory effect on the growth of several tumor types in nude mice. Since then, anti-VEGF therapies to target angiogenesis have gained further attention. Investigators have reported inhibited tumor growth with other anti-VEGF applications including anti-sense nucleotides, anti-receptor antibodies, soluble VEGF receptors, and a retrovirus-delivered Flk-1 mutant.^[46-49] A recent phase three FDA trial demonstrated improved survival when patients with metastatic colorectal cancer were treated with a humanized monoclonal anti-VEGF antibody (bevacizumab) in combination with standard chemotherapy (irinotecan, 5-FU, and leucovorin), resulting in FDA approval. The FDA has also approved its use in non-small cell lung cancer. Bevacizumab is being tested in several other tumor models with and without adjunctive traditional chemotherapy agents.

Anti-angiogenic therapies have targeted the normal endothelial cell's ability to respond to angiogenic factors, either by primarily inhibiting endothelial cell proliferation or by using antibodies to prevent angiogenic factors from activating endothelial cells^[50]. However, some investigators have observed resistance to this therapy due to the ability of tumor cells to upregulate the expression of VEGF and thereby negate the anti-angiogenic effect^[51]. There is further evidence suggesting that high tumor levels of VEGF may promote tumor survival^[52].

One potential anti-angiogenic strategy is to silence gene expression of angiogenic factors at the mRNA level.

While antisense oligonucleotides have been used for this strategy, the high concentration required and non-specific effects have limited this approach. First described in *C. elegans*, RNA interference (RNAi) is a process in which double stranded RNA (dsRNA) is processed by the enzyme Dicer, resulting in short interfering RNAs (siRNA) 21-25 nucleotides in length^[53,54]. These siRNA molecules are incorporated into an RNA-induced silencing complex (RISC) that binds complementary target mRNAs, leading to their degradation^[55,56].

There is great potential for using RNAi technology in therapeutic applications that target critical signaling pathways involved in cancer, due to its high specificity for targeted genes and its potency at low concentrations. However, relatively few studies have examined the use of RNAi technology to suppress VEGF production.

In 2003, three reports proved the concept. Filleur and colleagues used a rat fibrosarcoma model and systemically delivered siRNA to achieve a 66% decrease in tumor size when compared to controls^[57]. Reich and colleagues used direct injection of anti-VEGF siRNA to decrease choroidal neovascularization in laser-induced retinal injury in the rat^[58]. A plasmid-based siRNA delivery system driven by a Pol III promoter was used by Zhang and colleagues to show isoform-specific knockdown of VEGF^[59].

These three experiments provided the conceptual basis for two further experiments to demonstrate the efficacy of RNAi inhibition of VEGF. In 2005, Yoon and colleagues injected HT1080 fibrosarcoma cells stably transfected with anti-vegf siRNA into the subcutaneous tissue of rats. They reported substantial decrease in tumor volume and vessel density in resected specimens^[60]. In the same year, Kwon and colleagues described a bi-level approach to silencing VEGF. In their experiment, they combined zinc finger-mediated repression of transcription with RNAi technology to achieve knockdown of VEGF of over 90%^[61].

The critical problem with RNAi-based therapy is one of delivery. Whereas *in vitro* transfection of cells is easily accomplished using lipid-based solution, *in vivo* targeting of the siRNA molecules to cancer cells is far more challenging. Systemic delivery of naked siRNA is problematic as the drugs localize poorly and the degree of dilution is often unacceptable^[62]. Though synthetic vectors offer several advantages such as their relative safety and the ease of incorporating target-specific ligands, there are significant barriers to their effective use that have not yet been overcome^[63]. Viral vectors may offer specific advantages as well^[64].

CONCLUSION

Although much has been learned about VEGF since its discovery in 1989, much more remains to be understood. First, the relevance of hypoxic stabilization of VEGF mRNA must be established in the settings of both neoplasia and normal physiologic conditions. Does our current understanding of hypoxic stabilization provide a sufficient explanation with regard to tumor angiogenesis? Are there other mitogenic factors that can overwhelm the cell's innate response to hypoxia? Second, the balance between

cap-dependent and IRES-mediated translation must be elucidated. What are the factors that mitigate the switch from cap-dependent to IRES-mediated translation? Finally, it is clear that post-transcriptional regulation of VEGF has profound clinical implications with regard to targeting tumor angiogenesis. Will RNAi technology prove to be an efficacious therapeutic modality? If so, how will we deliver the drug to achieve a safe, durable, and clinically significant outcome? It will be a challenge for coming years to determine how best to exploit these molecular mechanisms for our patients' benefit.

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Hereditary non-polyposis colorectal cancer: The rise and fall of a confusing term

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Abstract

The term Hereditary Non-Polyposis Colorectal Cancer (HNPCC) is a poor descriptor of the syndrome described by Lynch. Over the last decade, the term has been applied to heterogeneous groups of families meeting limited clinical criteria, for example the Amsterdam criteria. It is now apparent that not all Amsterdam criteria-positive families have the Lynch syndrome. The term HNPCC has also been applied to clinical scenarios in which CRCs with DNA microsatellite instability are diagnosed but in which there is no vertical transmission of an altered DNA mismatch repair (MMR) gene. A term that has multiple, mutually incompatible meanings is highly problematic, particularly when it may influence the management of an individual family. The Lynch syndrome is best understood as a hereditary predisposition to malignancy that is explained by a germline mutation in a DNA MMR gene. The diagnosis does not depend in an absolute sense on any particular family pedigree structure or age of onset of malignancy. Families with a strong family history of colorectal cancer that do not have Lynch syndrome have been grouped as 'Familial Colorectal Cancer Type-X'. The first step in characterizing these cancer families is to distinguish them from Lynch syndrome. The term HNPCC no longer serves any useful purpose and should be phased out.

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INTRODUCTION

A fundamental concept in the successful practice of medicine is the recognition of a disease as a distinct entity defined by a clearly recognizable set of clinico-pathological features. Only by recognizing and diagnosing a specific disease is it possible to develop and apply an effective policy of disease management. The recognition of a distinct clinico-pathological entity does not depend on a full understanding of the underlying etiology providing that the appearance and natural history of a disease are sufficiently characteristic to set it apart from all other diseases. Many forms of cancer would fall into this category. The same arguments apply to complex syndromes, though many of these are in fact explained on the basis of a specific and heritable genetic abnormality.

A good example of a hereditary cancer syndrome due to a single gene defect is the autosomal dominant condition familial adenomatous polyposis (FAP) caused by germline mutation of the tumor suppressor gene *APC*^[1]. For many years Henry Lynch and associates drew attention to a second form of familial colorectal cancer (CRC)^[2,3]. Initially, this was believed to encompass two separate syndromes. Lynch I syndrome (or Hereditary Site-Specific Colon Cancer) applied to families with colon cancer only, while Lynch II syndrome (or Cancer Family Syndrome) included families with extra-colonic malignancies in addition to colon cancer. In 1985 Lynch introduced the term 'hereditary non-polyposis colorectal cancer' (HNPCC) to encompass Lynch syndromes I and II^[4]. In 1991 there was further unification with the inclusion of the Muir-Torre syndrome (CRC with associated sebaceous neoplasms) within HNPCC^[5]. Lynch viewed HNPCC as a syndrome characterized by an autosomal dominant pattern of inheritance, early onset of malignancy with a predilection for the proximal colon, multiple CRCs, the absence of premonitory lesions (e.g. adenomas), and the occurrence of cancer in certain extracolonic sites, notably endometrium and ovary.

The concept of a second major form of familial CRC to rival FAP was received with general skepticism, being either ignored, dismissed as an exceedingly rare disease or the chance clustering of a common disease, or interpreted as the result of a shared environmental exposure^[6]. A breakthrough came with the publication of

a series of clinico-pathological investigations conducted in Finland that fully supported the observations of Lynch^[7,8]. It was also shown that CRCs associated with HNPCC had particular histological features^[9,10]. These included poor differentiation, abundant mucin secretion and marked lymphocytic infiltration. Additionally, CRCs developed within adenomas and it was inferred that HNPCC adenomas were more likely to undergo malignant transformation and within a shorter timeframe than conventional adenomas^[9,11,12].

By the 1990s, cancer family clinics and registries for hereditary malignancy were beginning to collect and describe families with the features identified by Lynch and others. In order to standardize clinical and basic research it was suggested in 1991 by the International Collaborative Group on HNPCC that families should only be described as having HNPCC if they met strict criteria, known as the Amsterdam criteria (Table 1)^[13]. Initially it was thought that the Amsterdam criteria would be highly specific for HNPCC. Nevertheless, the term HNPCC and the associated diagnostic criteria soon generated problems and confusion that have continued to this day.

THE TERM HNPCC

The term HNPCC implies a familial aggregation of CRC developing in the absence of polyps. However, patients with HNPCC have adenomas that may sometimes be multiple and frequently present with extra-colonic malignancy. For example, a patient with HNPCC could present with endometrial cancer, multiple adenomas and no family history of cancer. By restricting the Amsterdam criteria to CRC, it rapidly became evident that the criteria lacked sensitivity. The Amsterdam II criteria (Table 1) were developed to allow particular cancers that presented in extra-colonic sites to be substituted for colorectal cancer, notably of endometrium, small intestine and pelviureter^[14]. When the genetic mechanism underlying HNPCC was eventually discovered (see below) it became apparent that even the Amsterdam II criteria lacked sensitivity. Clinical definitions were therefore relaxed in a variety of ways in different geographical regions in order to avoid missed diagnoses^[15-18]. Indeed, single instances of HNPCC might be found as a consequence of new mutation, non-penetrance within small families, denial of family history, non-paternity, or adoption. These new definitions of HNPCC were not used merely to standardize research but were also employed as diagnostic tools for labeling families as having HNPCC. In summary, the term HNPCC was not only a misleading descriptor but came to encompass multiple clinical definitions while nevertheless being applied to families as a specific diagnostic label.

DNA MISMATCH REPAIR

In 1993, soon after the introduction of the Amsterdam criteria, the underlying mechanism for HNPCC was discovered. Most examples were shown to be caused by germline mutation in one of two DNA mismatch repair (MMR) genes: *MSH2* or *MLH1*^[19-22]. *MSH6* was implicated less frequently but showed a stronger association with

Table 1 Amsterdam I and II criteria and Bethesda (revised) guidelines

Term	Criteria	Reference
Amsterdam I ¹	Three 1 st degree relatives with CRC Two generations affected One family member below age 50 yr Exclude familial adenomatous polyposis	Vasen <i>et al</i> , 1991 ^[13]
Amsterdam II ¹	As for Amsterdam I except that CRC may be substituted by cancer of endometrium, small bowel, or pelviureter	Vasen <i>et al</i> , 1999 ^[14]
Bethesda (revised) ²		Umar <i>et al</i> , 2004 ^[14]
1	CRC below age 50 yr	
2	Multiple CRC or HNPCC-related cancers ³	
3	CRC with MSI-related histology ⁴ , < 60 yr	
4	CRC or HNPCC-related cancer in at least one 1 st degree relative, < 50 yr	
5	CRC or HNPCC-related cancer in at least two 1 st or 2 nd degree relatives, any age	

¹All criteria must be met; ²Any criterion (1-5) can be met; ³Includes cancer of endometrium, small bowel, pelviureter, biliary tract, stomach, ovary, pancreas, and brain (mainly glioblastoma multiforme); ⁴Tumor infiltrating lymphocytes, Crohn-like reaction, mucin/signet ring cell differentiation, medullary growth pattern.

endometrial cancer^[23]. The MMR genes were shown to be tumor suppressor genes. Following inactivation of the wild-type allele, MMR proteins would no longer be expressed resulting in a failure to repair DNA mismatches occurring as spontaneous errors during DNA replication^[24]. This failure applied most particularly within non-encoding tracts of repetitive DNA called microsatellites but could also disrupt tumor suppressor genes with short repetitive tracts within their encoding regions, for example (and in decreasing order of frequency) *ACVR2*, *PTHLH*, *TGFbetaRII*, *MARCKS*, *MSH3*, *TCF4*, *RAD50*, *CASP5*, *BAX*, *RIZ*, *MBD4*, *MSH6*, *BLM*, *IGF2R*, *PTEN*, *AXIN2*, *WISP3* and *CDX2*^[25]. Deficiency of MMR therefore resulted in the distinct phenotype described as the mutator phenotype or, more specifically, as DNA microsatellite instability (MSI)^[26,27].

In addition, therefore, to its distinctive clinico-pathological features, the diagnosis of HNPCC could now also be suggested through the demonstration of DNA MSI and/or through the loss of expression of DNA MMR proteins, once monoclonal antibodies to these proteins became commercially available^[28,29]. The corollary that MMR deficiency was a specific biomarker for the syndrome was not however correct^[30]. This is because one of the DNA MMR genes, namely *MLH1*, may be inactivated through hypermethylation of its promoter region^[31]. Through this acquired epigenetic mechanism most CRCs with MSI present sporadically and not as a result of a germline mutation. Nevertheless, in families with the clinico-pathological features of HNPCC, the demonstration of MMR deficiency in one or more tumors is a very strong indicator of a germline mutation in a MMR gene (though not 100% specific, see below). In

summary, it was now possible to precisely demarcate and therefore diagnose HNPCC on the basis of the distinctive combination of clinical, pathological and molecular phenotype.

LACK OF SPECIFICITY OF CLINICAL CRITERIA FOR HNPCC

It was initially assumed that the Amsterdam criteria would be highly specific but not necessarily sensitive for HNPCC. However, the advent of testing for MSI showed that this assumption was not correct. In families meeting the Amsterdam criteria but in which CRCs did not show evidence of MSI, the classical clinical and pathological features of HNPCC were lacking^[32]. Compared with Amsterdam criteria families in which CRCs showed MSI, the non-MSI-positive families comprised fewer affected subjects and CRCs were less likely to be proximally located, show mucinous or poor differentiation, be associated with positive lymph nodes, or have diploid DNA content^[32]. CRCs were also less likely to be multiple or to present at a young age^[32]. Additionally, when subjects from non-MSI-positive families were colonoscoped, they were more likely to have adenomas but the adenomas were less likely to be advanced than in MSI-positive families^[33]. Despite these different clinicopathological findings, families continued to be diagnosed as having HNPCC merely on the basis of meeting the Amsterdam or similar clinical criteria^[34-36].

At this point the question arises whether the term 'HNPCC' is the most appropriate label for a specific hereditary cancer syndrome caused by a germline mutation in a DNA MMR gene. Henceforth, for the purposes of this discussion and in accordance with a recent suggestion^[6], the term 'Lynch syndrome' will be applied to the specific form of hereditary malignancy caused by germline mutation of a DNA mismatch repair gene. The term 'HNPCC' will be used merely to indicate that certain clinical criteria suggestive of a hereditary basis for CRC (excluding FAP) have been met. Families meeting one of the several clinical definitions of HNPCC may or may not have Lynch syndrome. There are three reasons why families may meet the Amsterdam criteria and yet not have the Lynch syndrome. In the first place, CRC is a common disease. Within a large sibship the finding of two affected siblings and one parent with CRC could occur by chance, even when one subject was diagnosed with CRC at the age of 49 years. Indeed, one would be appropriately skeptical of the diagnosis of Lynch syndrome if the other two affected subjects were diagnosed in their seventies or eighties. The labeling of a family with the diagnosis of Lynch syndrome on the basis of the preceding findings would be unwise, even though no 'rule' is being broken. A second reason is the possible clustering of low- or intermediate-risk genes within a family. The third reason is the possibility of family clusters occurring through known (e.g. attenuated FAP^[37] or *MYH* polyposis^[38]) or unknown high-risk genes. Although the lack of specificity of the Amsterdam criteria was first mooted in 1995^[32], recognition that families meeting the criteria are clinically heterogeneous has come about only comparatively

recently. For example, four recent studies have highlighted the lack of features of Lynch syndrome in Amsterdam criteria positive families that lack evidence of DNA MMR deficiency^[39-42]. Features of particular note are the older age of onset of CRC, the reduced penetrance among relatives, the lack of predilection for the proximal colon, the lack of lymphocytic infiltration, the lack of extra-colonic malignancies, and the higher frequency of colorectal adenoma^[39-42].

DIAGNOSIS AND MANAGEMENT OF LYNCH SYNDROME

The discussion above has outlined how over the last decade the Amsterdam or similar clinical criteria have often been the mainstay in reaching a purported diagnosis of Lynch syndrome. While larger, multi-case families with the classical spectrum of early onset colorectal and extra-colonic malignancies were probably diagnosed correctly in most cases using this approach, the diagnosis is less likely to have been correct in families meeting only the minimum set of clinical criteria. The effective management of Lynch syndrome includes informing and counseling affected and at-risk subjects, but this can only happen after the correct diagnosis has been reached and not before. Fortunately, a reliable working diagnosis of Lynch syndrome can usually be reached through the analysis of tissue samples prior to the discovery of a pathogenic germline mutation. Apart from confirming the diagnosis of cancer, evaluation of tissue samples provides both morphological and molecular evidence of tumorigenesis driven by defective DNA mismatch repair, the molecular hallmark of Lynch syndrome. The use of immuno-histochemistry to detect loss of expression of DNA MMR proteins can even identify the likely genetic cause and thereby simplify the ultimate diagnostic step of genetic screening^[28,29].

As noted above, the Amsterdam criteria were established to provide a standard definition of HNPCC presenting within the setting of high-risk cancer family clinics and registries. The Bethesda guidelines were developed as a guide for testing MMR status in CRCs that presented in the population setting (Table 1)^[43,44]. Family history, age at onset of malignancy, and pathology features were utilized as independent markers and in a way that would not only identify new cases of possible Lynch syndrome but would also exclude late-onset CRCs with MSI. The latter would in most instances be sporadic MSI-positive CRCs explicable by age-related methylation of *MLH1*. On this basis, the utilization of the Bethesda guidelines in order to diagnose Lynch syndrome should be more sensitive than the Amsterdam criteria and, through the inclusion of MMR testing within the algorithm, more specific^[45,46].

The Bethesda guidelines highlight the importance of pathology review. This has not always been used in achieving a diagnosis of Lynch syndrome despite the fact that the attempt to arrive at an exclusively clinical definition of Lynch syndrome has been described as a 'search for the impossible'^[47]. There are three reasons for this over-reliance on limited clinical features: (1) The Amsterdam criteria were initially thought to be highly stringent and

therefore specific for Lynch syndrome, (2) It is easier and cheaper to reach the diagnosis using clinical features alone, and (3) It has been argued that both MSI testing and immuno-histochemistry are genetic tests that should not be undertaken without the consent of the patient. With respect to the last, MSI testing and immuno-histochemistry are diagnostic tests capable of demonstrating an acquired and tumor-specific alteration that is indicative of but not conclusive with respect to germline status. Mismatch repair deficiency may be acquired exclusively at the somatic level, for example through methylation of a DNA mismatch repair gene (see above). Like all diagnostic tests of tumor phenotype, the results of MSI testing do not stand alone but must be interpreted in the light of all the available clinical and pathological features.

Because the term HNPCC was introduced as an alternative to the eponymous Lynch syndrome before that condition was fully characterized and explained on the basis of a set of genes with closely related function, it came to be applied in an overly broad sense to any form of CRC that appeared to be inherited but was not FAP (or the other rarer precancerous polyposes). However, it is not possible to use a term that has different and mutually exclusive meanings without generating confusion. The following two sections describe additional scenarios in which the term HNPCC (implying Lynch syndrome) has been used inappropriately.

GERMLINE HEMI-ALLELIC METHYLATION OF *MLH1*

Extrapolating from the Bethesda guidelines (see above), an early-onset CRC that shows MSI is likely to occur in the setting of Lynch syndrome. In 2002, Gazzoli *et al* identified an early onset CRC in which one *MLH1* allele showed methylation^[48]. Intriguingly, the same allele was also methylated in the subject's lymphocytes. By elegantly exploiting the existence of a common polymorphism in the promoter region of *MLH1*, Gazzoli *et al* were able to show that the second or wild-type *MLH1* allele had been lost in the CRC. They therefore introduced the concept of germline hemi-allelic methylation of *MLH1* as a cause of HNPCC^[48]. However, they were skeptical of the possibility that a methylated allele could be transmitted vertically from an affected subject to offspring and suggested that the phenomenon was both rare and sporadic. Miyakura *et al* discovered four additional examples of early onset MSI-positive CRC associated with germline hemi-allelic methylation of *MLH1*^[49]. Although the patients were ascertained through cancer family clinics they did not have family histories suggestive of Lynch syndrome. They were merely young and some were affected with multiple tumors consistent with Lynch syndrome. Again, Miyakura *et al* did not infer that germline hemi-allelic methylation of *MLH1* could be transmitted vertically^[49].

Suter *et al* arrived at a different conclusion with respect to germline hemi-allelic methylation of *MLH1* (which they termed 'epimutation')^[50]. They documented two additional subjects carrying an *MLH1* epimutation who also met clinical criteria indicative of a diagnosis of

HNPCC. Additionally, the epimutation was present in spermatozoa of one of the affected subjects. The latter finding was not only consistent with a germline defect but also provided evidence for transmission of the defect to offspring. The authors therefore advanced the concept of *MLH1* epimutation as a new cause of HNPCC (implying Lynch syndrome) in which there was vertical transmission of a methylated *MLH1* allele^[50]. Nevertheless, it may be questioned if epimutations can in fact be inherited. While germline hemi-allelic methylation was indeed demonstrated in single members of two families that met certain clinical criteria for HNPCC, this is hardly surprising since the search for the epimutation was conducted exclusively in members of families registered in cancer family clinics. This ascertainment bias aside, it is now abundantly clear (see discussion above) that when a family happens to meet a particular clinical definition of HNPCC this does not automatically prove the existence of a heritable cause of cancer that is due to an altered DNA MMR gene^[32,39-42]. It is true that one of the affected subjects showed methylation of *MLH1* within spermatozoa, but this was in less than 1% of spermatozoa^[50]. Even if such a sperm succeeded in fertilizing an ovum, subsequent clearance of methylation during early embryogenesis would negate the effects of vertical transmission of the affected allele.

The same authors who advanced the concept of the heritability of *MLH1* epimutation subsequently demonstrated the *de novo* origin of germline hemi-allelic methylation of *MLH1* in a male subject who was shown to have inherited the methylated allele from his mother in whom the same allele was not methylated^[51]. These authors nevertheless continued to claim that *MLH1* epimutation was 'weakly' heritable^[51]. The fact that genetic alterations may be passed from parent to child and result in syndromes of familial cancer associated with early onset of disease and multiple neoplasia is a dismal truth for affected families and presents multiple problems with respect to the provision of medical solutions that are ethical, compassionate and effective. Therefore, the labeling of a form of cancer as heritable should be backed up by good evidence. The existing evidence does not support the heritability of germline hemi-allelic methylation of *MLH1*.

SERRATED POLYPS, DNA METHYLATION AND HNPCC

In 1997 a family was described as having HNPCC but did not meet the Amsterdam I or II criteria^[52]. There were four siblings in a sibship of six with a total of eight CRCs between them. The initial CRCs presented at the ages of 44, 27, 44 and 55 years (eldest to youngest sibling respectively) and an offspring of the second eldest developed a gastric cancer at the age of 29 years. Gastric cancer is linked to Lynch syndrome but is not included in the Amsterdam II criteria. MSI testing using four markers was initially performed on the CRCs from the eldest and third eldest siblings and all four markers were unstable in both CRCs^[52]. Overall, therefore, the features were consistent with a diagnosis of Lynch syndrome. Only one of the colectomy specimens, a right

hemicolectomy performed on the youngest sibling for two metachronous CRCs, was available for detailed study at the time of surgery. The pathology was atypical for Lynch syndrome insofar as there were five tubular adenomas, seven hyperplastic polyps and seven mixed polyps. The hyperplastic polyps and mixed polyps were noted to be larger than the adenomas^[52]. In retrospect, the correct diagnosis would now be hyperplastic polyposis^[53]. The smaller CRC showed conspicuous lymphocytic infiltration, instability at three of three microsatellite markers, and was arising from a mixed polyp. The large CRC was mucinous and showed instability at two of three microsatellite markers. One of the offspring of this subject had developed a tubulovillous adenoma and two hyperplastic polyps at around the age of 40 years and a second was found to have multiple hyperplastic polyps after the report was published. Overall, it was inferred that this family had Lynch syndrome but that there was a modifying genetic factor in the youngest sibling giving an unusual phenotype^[52]. Nevertheless, no germline mutation in a DNA mismatch repair gene has been found in this family.

Now, in retrospect, it is possible to suggest a simpler and more plausible mechanism underlying this Lynch syndrome-like family. In 2005, a paper described a series of eleven Lynch syndrome-like families in which some CRCs were MSI-high but others had low-level MSI or were microsatellite stable (MSS)^[54]. A more consistent finding across the CRCs and polyps in these 'MSI-variable' families was mutation of the oncogene *BRAF* and methylation of *MINT31*^[54]. In addition, many subjects had advanced serrated polyps and two had hyperplastic polyposis. Based on these observations it was suggested that an inherited predisposition to acquired DNA methylation within somatic tissues could give rise to a 'serrated pathway syndrome'^[54]. Should *MLH1* be implicated then one might observe the development of CRCs that were MSI-high. This would only apply to a subset of CRCs, but could by chance, as in the case of the family described in 1997^[52], affect all CRCs tested within a single family.

What is the evidence for an inherited predisposition to CRCs with DNA methylation? In a family cancer clinic-based study that excluded families with the Lynch syndrome, subjects with CRCs showing the CpG island methylator phenotype (CIMP) had a 13-fold increased risk of having a first degree relative with cancer (not necessarily CRC) as compared with subjects without CIMP-positive CRC^[55]. A hospital-based study could not confirm this finding but removed families meeting a clinical definition for HNPCC^[56]. The latter exclusion clearly introduced a major bias since not all families meeting a clinical definition for HNPCC in fact carry a germline defect in a DNA MMR gene (see above). This is another example of how a loose definition of HNPCC can be confounding.

An inherent difficulty in establishing whether genetic factors may explain CIMP is the lack of an agreed definition of CIMP. It is clear, however, that mutation of *BRAF* co-segregates with extensive CIMP and may therefore be used as a surrogate for high-level CIMP. In a large population-based study, CRCs were stratified on the basis of *BRAF* mutation and DNA MSI status^[57]. In the larger subset of MSS CRCs, the odds ratio for having a

positive family for subjects with *BRAF* mutation-positive CRCs was 4.23 (95% CI = 1.65-10.84) (as compared with subjects with *BRAF* mutation-negative CRCs). The same finding was not observed in patients with MSI-high CRCs^[57]. However, this is not surprising since the subjects with MSI-high CRCs that lacked *BRAF* mutation were relatively young and many would be expected to have Lynch syndrome.

In summary, it is likely that genetic mechanisms will be found to at least partially explain the evolution of CIMP-positive CRCs and to account for a subset of families that may mimic Lynch syndrome but in whom both colorectal polyps and cancers show extensive DNA methylation and frequent mutation of *BRAF*.

LYNCH SYNDROME VARIANTS

The more clearly one defines Lynch syndrome, the simpler it becomes to distinguish the syndrome from clinical scenarios that provide close mimicry. In the preceding two sections we encountered different clinical situations in which there was diagnosis of CRCs with DNA MSI and an inappropriate label of HNPCC. The essential feature of Lynch syndrome is that it is a *hereditary* disorder that is explained by a *germline mutation in a DNA MMR gene*. DNA MSI is a useful diagnostic hallmark but is not requisite for the diagnosis. Some mutations in DNA MMR repair genes may be tumorigenic but may not result in the mutator phenotype^[58]. DNA MMR repair genes are multifunctional and certain mutations may be deleterious because they disrupt cell-cycle checkpoint control and/or apoptosis^[59]. These variants may be associated with lower penetrance and a milder clinical phenotype but should probably be regarded as variants of Lynch syndrome. Mutations in the DNA MMR gene *PMS2* may result in a syndrome that is inherited as an autosomal recessive trait^[60]. The combination of very early onset malignancy involving brain, colon and other sites has been described as Turcot syndrome and linked with bi-allelic mutation of *PMS2* in a small number of families^[60,61]. Drawing a distinction between Turcot syndrome and Lynch syndrome becomes difficult given the fact that *PMS2* mutation has also been linked with autosomal dominant patterns of inheritance^[62,63] while certain *MLH1*, *MSH2* and *MSH6* mutations may give rise to an autosomal recessive trait^[61,64]. Individuals with homozygous MMR gene mutations may show features of neurofibromatosis with café-au-lait spots. This has been associated with somatic mutation of the neurofibromatosis type-1 gene^[65]. The preceding examples could be considered as variants of Lynch syndrome. However, clinical scenarios in which CRCs with MMR deficiency occur in the absence of a vertically transmissible genetic alteration in a MMR gene should not be termed Lynch syndrome.

CONCLUSION

The term HNPCC is a poor descriptor of the syndrome described by Lynch. Over the last decade, the term has been applied as a specific diagnostic label to families meeting a multiplicity of clinical criteria. A subset of

Table 2 Conditions and clinical scenarios that may mimic Lynch syndrome

Attenuated Familial Adenomatous Polyposis ^[37] (polyps may not be numerous)
MYH polyposis ^[38] (polyps may not be numerous)
Juvenile polyposis ^[66] (polyps may not be numerous and may be adenomatous)
Germline mutation of <i>TGFbetaRII</i> ^[67] and <i>AXIN2</i> ^[68] (is not associated with DNA MSI)
Hereditary mixed polyposis syndrome ^[69] (polyps may not be numerous and may be adenomatous)
Hyperplastic polyposis ^[53] (polyps may not be numerous and may be adenomatous)
Serrated Pathway Syndrome ^[54] (polyps may not be numerous and may be adenomatous and CRCs may show DNA MSI)
Germline hemi-allelic methylation of <i>MLH1</i> ^[48-51] (multiple, early onset cancers with Lynch syndrome spectrum and having DNA MSI)
Lynch syndrome variants ^[61,65] (autosomal recessive Turcot syndrome due to <i>PMS2</i> mutation)
Familial Colorectal Cancer-Type X ^[40] (Amsterdam criteria met but Lynch syndrome and conditions listed above have been excluded)

these families will not carry a germline mutation in DNA MMR gene and will not show the clinical and pathological phenotype associated with the Lynch syndrome. Such families may meet or even exceed the Amsterdam criteria despite lacking evidence for a germline defect in a DNA mismatch repair gene. It has been suggested that these families be grouped as 'Familial Colorectal Cancer Type-X'^[40]. The term HNPCC has also been applied to clinical scenarios in which CRCs with DNA MSI are diagnosed but in which there is no vertical transmission of an altered DNA MMR gene. A diagnostic term that has multiple mutually incompatible meanings is problematic. This becomes particularly evident when extrapolating from a diagnostic label to the management of an individual family.

The Lynch syndrome is best understood as a *hereditary predisposition to malignancy* that is explained by a *germline mutation in a DNA MMR gene*. The diagnosis does not depend in an absolute sense on any particular family pedigree structure or age of onset of malignancy. If present, these are merely useful clinical pointers that have been accorded undue importance when considered in isolation of other facts. No simple set of clinical criteria can serve as a diagnostic label for Lynch syndrome. At the same time, the careful appraisal of clinical, pathological and molecular features can achieve an accurate working diagnosis prior to the demonstration of a pathogenic germline mutation in a DNA mismatch repair gene. To paraphrase a recent recommendation with respect to the term HNPCC: 'Clarification of the genetic basis and full phenotypic expression of this disease mandates a more clinically useful name that gives consideration to non-colonic cancers and unifies the diagnosis around germline mutation in a DNA MMR gene. The term Lynch syndrome is proposed for the autosomal dominant disease caused by a germline mutation in a DNA MMR gene'^[6]. However, in adopting the term Lynch syndrome in place of HNPCC one must also distinguish Lynch syndrome from the numerous clinical scenarios that give rise to close mimicry. By separating and naming these clinical

entities (Table 2) one can begin to undo the considerable confusion that has been generated by the arbitrary use of the imprecise term HNPCC.

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Insights on augmenter of liver regeneration cloning and function

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Abstract

Hepatic stimulator substance (HSS) has been referred to as a liver-specific but species non-specific growth factor. Gradient purification and sequence analysis of HSS protein indicated that it contained the augmenter of liver regeneration (ALR), also known as hepatopoietin (HPO). ALR, acting as a hepatotrophic growth factor, specifically stimulated proliferation of cultured hepatocytes as well as hepatoma cells *in vitro*, promoted liver regeneration and recovery of damaged hepatocytes and rescued acute hepatic failure *in vivo*. ALR belongs to the new Erv1/Alr protein family, members of which are found in lower and higher eukaryotes from yeast to man and even in some double-stranded DNA viruses. The present review article focuses on the molecular biology of ALR, examining the ALR gene and its expression from yeast to man and the biological function of ALR protein. ALR protein seems to be non-liver-specific as was previously believed, increasing the necessity to extend research on mammalian ALR protein in different tissues, organs and developmental stages in conditions of normal and abnormal cellular growth.

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Key words: Hepatic stimulator substance; Augmenter of liver regeneration; Liver regeneration; Molecular biology

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INTRODUCTION

In 1975, La Brecque and Pesch first referred to the existence of a polypeptide, named hepatic stimulator substance (HSS), in the liver cytosol of weanling or adult partially hepatectomized rats^[1]. HSS was able to specifically stimulate hepatocyte proliferation and support liver regeneration in an organ specific but species non-specific manner^[1-2]. Since then a number of studies examined HSS levels of toxicity on animal models of liver regeneration after partial hepatectomy and post induced liver injury *in vivo*^[3,4] and *in vitro*^[5]. Subsequently, the effect of this growth factor in acute^[6-11] and chronic^[12] liver injury and decreased hepatocyte proliferative capacity^[13,14] has been reported in different animal models. A novel growth factor, obtained through progressive purification (831 000x) of the crude HSS extract, was named Augmenter of Liver Regeneration (ALR)^[15-20]. ALR, as well as HSS, did not affect quiescent hepatocytes but enhanced the liver proliferative response to hepatectomy in rats^[1,16,21] and dogs^[22] and prevented atrophy increased cell renewal caused by portacaval shunt (Eck's fistula) in dogs^[23,24]. ALR recently has been the subject of intense investigation. Several different approaches have led to the identification of the respective genes from yeast, rat, mouse, and human. This review deals with the molecular biology of ALR, examining cloning of the ALR gene and its protein product from yeast to man. The biological function of this protein product is also described.

ALR GENE AND PROTEIN FROM YEAST TO MAN

Yeast scERV1

The yeast scERV1 gene was the first and best-characterized gene of a new family found in a large number of lower and higher eukaryotes^[25-28] and in the genome of some double-stranded DNA viruses^[29-31]. This gene encodes a small protein of 189 amino acids (22 ku) having a complex influence on different aspects of mitochondrial biogenesis^[32-34] and is essential for the survival of yeast^[28]. Further studies have shown that the protein (Erv1p) was essential for normal mitochondrial morphology and for the stable maintenance of these organelles. In addition, Erv1p was identified as the first FAD-linked sulfhydryl oxidase from yeast^[34]. The enzymatic activity was located at the 15 ku carboxy-terminal domain of the Erv1p with the conserved Cys-X-X-Cys sequence motif (CXXC motif). This frag-

ment was able to bind FAD and to catalyze the formation of disulfide bonds but was no longer able to form dimers like the complete protein^[35]. Last but not least, analysis of the complete genome sequence from *S. cerevisiae* revealed a second yeast gene with homologies and structural similarities to scERV1. This gene was termed scERV2. Its protein product (Erv2p) had a length of 196 amino acids and exhibited 30% identical amino acid residues with Erv1p in the highly conserved carboxy-terminal part of the polypeptides. However, the complex expression pattern and the high degree of variability found in the amino-terminal regions of the scERV1 and scERV2 proteins, as well as the differences found between them, indicated that the gene products might have diverse functions at different locations and critical times of cellular development^[30].

Rat ALR

Hagiya *et al* in 1994, first discovered and cloned ALR in rat and then in human and mouse, proposing also its identity with HSS or hepatopoietin (HPO)^[25]. They cloned and sequenced the rat ALR gene, which was considered to encode rat HSS^[25]. They also presented evidence that ALR protein (Alrp) was acting as a homodimer and reported that the expression of the 1.2 kb gene transcript was elevated in rat testis and liver. The 1.2 kb cDNA included a 299 bp 5' untranslated region, a 375 bp coding region and a 550 bp 3' untranslated one. The rat Alrp consisted of 125 amino acids and its molecular weight was calculated to be 15 ku, which is consistent with its electrophoretically determined molecular weight under reducing conditions. The molecular weight of the purified native Alrp was calculated to be 30 ku under nonreducing conditions. The 125 amino acid sequence deduced from the rat ALR cDNA presented as 50% homologous to the polypeptide encoded by the scERV1, which is essential for oxidative phosphorylation, vegetative growth and life of the yeast *S. cerevisiae*^[25]. The same investigators, in supplementary experiments, found a single nucleotide (G) insertion at cDNA position 266, which did not alter the results of recombinant Alrp of 125 amino acids that was originally identified. On the other hand, additional G engendered two other in-frame ATG initiation sites, which were 5' to the initiation site of the Alrp they had previously reported, thus raising the possibility of additional ALR variants^[36]. Furthermore, the cloning and sequence analysis of rat genomic DNA of ALR revealed that the gene consisted of 3 exons and 2 introns and was 1508 nt long^[37]. An ALR pseudogene was also found in the rat genome, implying the existence of an ALR multigene family. The length of the pseudogene was calculated to be 442 nt^[37]. The amino acid sequence homology between the rat ALR and its pseudogene proved to be 88.8%^[38]. The crystal structure of recombinant rat Alrp has been determined to 1.8 Å. The structure revealed a unique FAD binding motif, a region containing side chain rings in a stacked parallel orientation, as has recently been reported in Erv2p, and an extensive salt bridges network being unique to ALR. The abundance of disulfide and salt bridges provides the Alrp molecule with a rigid structure and probably accounts for Alrp's thermostability and resistance to some denaturants^[39].

Mouse ALR

The mouse ALR gene was cloned and characterized by Giorda *et al* in 1996^[26]. It was shown that the protein coding portion of the mouse ALR gene contained 3 exons. The first of them consisted of the 5' untranslated sequence and the initial 18 bases after the ATG translation initiation codon. The second exon contained 198 bases and the third one consisted of the remaining portion of the protein coding sequence. Moreover, the ALR gene mapped to mouse chromosome 17, in a region syntenic with human chromosome 16^[26].

Human ALR

Giorda *et al* also published the cDNA sequence of human ALR^[26]. At the same time, Yang *et al* cloned the cDNA of human HPO by functional screening of a fetal liver cDNA library demonstrating that human HPO was identical to human ALR^[40]. The cDNA of human ALR encoding 125 amino acids presented identity of 87% with rat ALR and of 42% to yeast scERV1^[40]. The human ALR gene was located on chromosome 16, at the cytogenetic band 16p13.3-p13.12, in the interval containing the locus for polycystic kidney disease (PKD1)^[27]. The cloning and sequence analysis of human ALR genomic DNA revealed that it consisted of 3 exons and 2 introns. It was 1813 nt long, coding a protein of 125 amino acid residues. A comparison of human and mouse genomic DNA demonstrated that the 3 exons were similar in length but different in their 5'-UTR, introns and 3'-UTR in length^[41,42]. Lu *et al* isolated a variant of ALR from hepatic-derived cells with a length of 205 amino acids, characterizing a novel isoform of HPO cDNA encoding a 205 amino acid open reading frame (ORF). This was named HPO-205 to distinguish it from the previously described HPO, which lacked the N-terminal 80 amino acids^[43].

Therefore, two forms of the human Alrp were found to be present in hepatocytes. The shorter protein consisted of 125 amino acids (15 ku) and lacked the amino terminus and the longer protein had 205 amino acids (23 ku). The 15 ku of Alrp existed only in the nucleus and the 23 ku Alrp was mainly located in the cytosol, probably because they are synthesized from the same mRNA using different initiation codons^[44]. Moreover, under normal conditions, the full-length 23 ku mammalian Alrp is predominant and localized in the mitochondrial intermembrane space^[45]. As a result, ALR could be dimerized at a protein level and its gene could be alternatively spliced at the transcriptional level. Both might contribute to the existence of various Alrp complexes in hepatocytes^[44].

Recent studies reported successfully cloning the genes encoding proteins that interact with Alrp, such as metallothionein and albumin, which might be related to the transportation process of Alrp in the circulation. Selenoprotein-P, an anti-oxidant protein, might also be related to Alrp function, as well as other ALR-binding proteins, such elongation factor 1- α , transitional endoplasmic reticulum ATPase, carboxypeptidase N 83 ku chain, complete factor H related 3, and NADP dehydrogenase, which are known to play important roles in energy metabolism^[46].

Table 1 Augmenter of liver regeneration from yeast to man

	Homo Sapiens (Human)	Mus Musculus (Mouse)	Rattus Norvegicus (Rat)	Saccharomyces cerevisiae (Yeast)
Description	Augmenter of liver regeneration (ALR)/growth factor, erv1 (<i>S. cerevisiae</i>)-like (augmenter of liver regeneration) (GFER)/hepatic regenerative stimulation substance (HSS)/hepatopoietin (HPO)	Augmenter of liver regeneration (Alr)/growth factor, erv1 (<i>S. cerevisiae</i>)-like (augmenter of liver regeneration) (Gfer)	Augmenter of liver regeneration (ALR)/growth factor, erv1 (<i>S. cerevisiae</i>)-like (augmenter of liver regeneration) (Gfer)	<i>Saccharomyces cerevisiae</i> ERV1 (scERV1)/flavin-linked sulfhydryl oxidase localized to the mitochondrial intermembrane space, has a role in the maturation of cytosolic iron-sulphur proteins; ortholog of human hepatopoietin (ALR)
Genomic Location	Chromosome: 16 Cytogenetic: 16p13.3-p13.12	Chromosome: 17 Cytogenetic: 17 A3.3	Chromosome: 10 Cytogenetic: 10q12 Genetic: 17 10.0 cM	Chromosome: VII
Peptide Length	205 aa (23 ku) 125 aa (15 ku)	198 aa	125 aa (15 ku)	189 aa (22 ku)
Protein Function	In liver regeneration and spermatogenesis	In liver regeneration and spermatogenesis	In liver regeneration and spermatogenesis	Essential for the biogenesis of mitochondria, cell viability and for vegetative growth
Protein Subunit	Homodimer/Heterodimer	Homodimer	Homodimer	Homodimer
Protein Similarity	Belongs to ERV1/ALR family	Belongs to ERV1/ALR family	Belongs to ERV1/ALR family	Belongs to ERV1/ALR family
Percentage of similarity to human		85.6% [The comparison with human is based on nucleic acid (GeneCard for GFER)] 87.20% [The comparison with human is based on amino acid sequence (UniGene)]	85.87% [The comparison with human is based on nucleic acid (GeneCard for GFER)] 85.60% [The comparison with human is based on amino acid sequence (UniGene)]	52.85% [The comparison with human is based on nucleic acid (GeneCard for GFER)] 46.85% [The comparison with human is based on amino acid sequence (UniGene)]
Subcellular Location	Nucleus (15 ku) Cytosol (23 ku) Mitochondrial intermembrane space (23 ku)	Mitochondrial intermembrane space	Cytosol Mitochondrial intermembrane space	Cytosol Mitochondrial intermembrane space

The ALR gene promoter has also been studied and might represent a novel architecture for core promoters. It was TATA-less and spanned position -54 to +42 relative to the transcriptional start point. Specifically, it did not contain the TATA box, a consensus A/T rich sequence [TATA (A/T) A (A/T)], which was located -25-30 nucleotides upstream of the transcriptional start site and was recognized by the TATA binding protein subunit of TFIID. TFIID is a transcription factor that was needed to bring about the initiation of transcription by RNA polymerase II and nucleated the formation of the pre-initiation complex. In addition, the core promoter of ALR consisted of a functional initiator (Inr) and three CTGGAGGC tandem repeat elements, which were found surrounding the Inr, with all of them participating in Inr-dependent transcription. The Inr contains a pyrimidine (Y) rich core sequence [YYA+1N (T/A) YY] encompassing the transcription site. The initiator flanking element, lying either upstream or downstream from the Inr, was found to be present in many Inr-containing genes. Both the initiator and at least one of the repeats were able to bind to specific nuclear factors^[47].

The similarities and differences between the structure and function of the ALR gene and its protein product in the four most important and closely related species (yeast, mouse, rat and human) are displayed in Table 1.

BIOLOGICAL FUNCTION OF ALRP

Characterization of human *Alrp* as a structural and functional homologue for yeast *Erv1p*

Alrp and *Erv1p* belong to a protein family, members of which are found in lower and higher eukaryotes from yeast to man^[25-28] and even on the genome of some double-stranded DNA viruses^[29-31]. They have essential functions in the biogenesis of mitochondria, the cell division cycle, and in the development of organs such as liver and testis in higher eukaryotes^[48]. The carboxy terminus of the human *Alrp* was characterized as a structural and functional homologue for yeast *Erv1p*^[27]. Especially, Lisowsky *et al* found four major similarities at the amino acid residues of carboxy termini of *Erv1p* and human *Alrp*, concluding a conservation of structure and function^[27]. However, Hofhaus *et al* showed that the conserved carboxy terminus of mammalian and yeast proteins were functionally interchangeable between distantly related species such as yeast and man^[49]. On the other hand, the amino terminal parts of the proteins displayed a high degree of variability and significant differences even among closely related species^[49]. Specifically, Hofhaus *et al* first revealed that the yeast protein contained a leader sequence for mitochondrial localization whereas the human protein is not detectable inside the mitochondria. Moreover, they noted that, in yeast only, one form of scERV1 was found

whereas in human cells two protein isoforms were present. Therefore, these two significant differences in the amino termini between Erv1p and human Alrp suggested that the human Alrp did not appear to be functional in yeast^[49].

The enzymatic activity of Alrp as sulfhydryl oxidase

The Alrp/Erv1p family belongs to flavin-linked sulfhydryl oxidase participating in disulfide bond formation^[35,50]. Lisowsky *et al* identified the enzymatic activity of rat and human Alrp as sulfhydryl oxidases by their ability to oxidize thiol groups in a protein substrate, the presence of FAD moiety in the carboxy terminal domain and the formation of dimers *in vivo*^[50]. In general, the sulfhydryl oxidase proteins contain a conserved CXXC motif in the carboxy terminal domain and a non-covalent FAD, which are vital for their catalytic activity^[51-53]. The conserved CXXC motif in the carboxy terminus most likely represents the redox-active site of the enzyme, sharing significant homologies with human growth regulator quiescin Q6^[52] and yeast Erv1p^[35]. It was also reported that the two vicinal cysteines directly interact with bound FAD for the redox reaction. Furthermore, sulfhydryl oxidases form dimers *in vivo* and catalyze the formation of disulfide bonds with the reduction of molecular oxygen to hydrogen peroxide according to the following reaction: $2RS-H + O_2 \rightarrow RSSR + H_2O_2$ ^[51-53].

An interesting new aspect for mitochondrial sulfhydryl oxidase activity and the formation of disulfide bonds was the recent finding of a direct correlation between the electron transport chain and the formation of disulfide bonds in *E. coli*^[54,55]. A very recent study on the fundamental behavior of the short form of the human Alrp suggested that this flavoenzyme might not necessarily function as sulfhydryl oxidase in the mitochondrial intermembrane space but might be coupled with the respiration chain *via* mediation of cytochrome *c* and without the generation of hydrogen peroxide observed in oxidase reactions. Thus, cytochrome *c* should be considered as a potential oxidant for ALR *in vivo*^[56].

The presence of a specific receptor for Alrp and modes of Alrp signaling

Wang *et al* showed the existence of an Alrp receptor on rat hepatocytes and human hepatoma cells with a molecular weight of about 75 ku, presenting with high affinity and specificity, reversibility and saturation^[57]. Alrp binds the receptor in cell membranes initiating a corresponding signal transduction pathway and mediating its biological function on hepatocytes. This function includes specific phosphorylation and dephosphorylation of important proteins in the process of liver regeneration^[57].

Yang *et al* showed that recombinant human HPO/Alrp (125 amino acids, 15 ku) could stimulate proliferation of hepatocytes as well as hepatoma cells *in vitro*, promoting regeneration and recovery of damaged hepatocytes and ameliorating acute hepatic failure *in vivo*. Such observations support the contention that Alrp is a hepatotropic growth factor^[58,59].

It was shown that HPO/Alrp was able to stimulate hepatocyte proliferation by two signaling pathways. In the

first pathway, extracellular HPO autocrined from hepatocytes and hepatoma cells activated the mitogen-activated protein kinase (MAPK) signaling pathway *via* binding to an ALR receptor. Specifically, Alrp triggered MAPK activation and proliferation in hepatoma cell lines through the induction of tyrosine phosphorylation of epidermal growth factor receptor (EGFR)^[60].

In the second signaling pathway, intracellular Alrp interacted with Jun activation domain-binding protein 1 (JAB1), triggering the activating protein-1 (AP-1) transcriptional activity in a MAPK independent manner, for immediate early response when its intracellular levels were increased with post-partial hepatectomy or liver injury^[61]. JAB1 is the fifth subunit of COP9 signalosome (CSN), being a co-activator of c-Jun/AP-1 transcription factor, which enhances the binding capacity of c-Jun-containing AP-1 complex to their DNA consensus sites and increasing the transactivation of an AP-1 dependent promoter. It was shown that Alrp enhanced the increased phosphorylation level of c-Jun through JAB1 but had no effect on the expression of transfected c-Jun or endogenous c-Jun N-terminal kinase or on phosphorylation of c-Jun N-terminal kinase^[61]. Furthermore, recent studies elucidated a novel relationship of intracellular Alrp with the whole protein complex CSN, suggesting a possible linkage between CSN and liver regeneration^[62]. As a consequence, the CSN complex could represent an intracellular signal platform, where signals from the extracellular or intracellular environment are coordinated with transcriptional activation and with the regulation of the related functions of cells.

Chen *et al* presented a molecular link between the enzymatic redox function of HPO/Alrp and its role as a cytokine^[63]. They displayed the necessity of cysteine residues in the CXXC catalytic centre for the intracellular potentiation of AP-1 activity. As mentioned before, Alrp interacted with JAB1 to trigger AP-1 transcription activity by phosphorylation of c-Jun in a MAPK independent fashion. This effect depended on the integrity of the CXXC enzyme active site, which could provide a novel intracellular signaling pathway shortcut by redox regulation. This implied that the enzymatic activity of Alrp might be a key regulator in the intracellular mediation of the AP-1 pathway through JAB1. On the other hand, they provided evidence that the extracellular cytokine activity of Alrp did not associate with the redox CXXC motif and consequently with its sulfhydryl oxidase activity^[63].

Recently, Li *et al* demonstrated that ALR and macrophage inhibitory factor (MIF) could be considered as a pair in the course of signaling transduction^[64]. Their results showed an interaction between ALR and MIF and led to the alteration of their effects on AP-1 activity. Especially, on the one hand, the binding of MIF to JAB1 was inhibited by ALR and on the other hand the potentiation of the ALR on AP-1 activity through JAB1 was inhibited by MIF. Thus, the activity of AP-1 may be determined by the coordination and balance between ALR and MIF concentration *in vivo*. This balance is crucial for the maintenance of both homeostasis and normal development of cells and tissues. In this sense, ALR serves as physiological counter-regulatory mediator that counteracts the suppressive effect of MIF^[64].

Alrp biological activity

Previous studies have demonstrated that different forms of Alrp may be associated with different localizations and cellular functions^[26,49,65]. Specifically, a 15 ku Alrp fragment appeared to regulate mitochondrial gene expression by inducing the transcription and translation of the nuclear-encoded mitochondrial transcription factor A (mtTFA)^[66]. Polimeno *et al* found that Alrp administration induced a significant increase in mitochondrial gene expression and of nuclear-encoded mtTFA. This increase was associated with enhanced cytochrome content and oxidative phosphorylation capacity of rat liver-derived mitochondria^[66]. It has also been suggested that hepatotrophic factors, such as Alrp, play an important role in liver regeneration and might mediate their activities by regional regulation of natural killer (NK) cells. However, NK cells in liver appeared to have specific cytotoxicity against regenerating hepatocytes. A recent study revealed that *in vivo* administration of Alrp induced inhibition of hepatic NK cytotoxic activities in the population of mononuclear leukocytes infiltrating rat liver but not in those derived from spleen or peripheral blood. These data suggested that Alrp might act as an immunosuppressant agent localized to the liver^[67]. Moreover, Tanigawa *et al* suggested that Alrp production in the liver was enhanced during acute liver disease, especially in the initial stage^[68]. It was also suggested that Alrp might protect against failure of regeneration by inhibiting hepatic NK cell activity in acute liver disease^[68]. It was also demonstrated that Alrp acted as an immunoregulator by controlling, through interferon-gamma (IFN- γ) levels, the mtTFA expression and lytic activity of liver-resident NK cells. The exogenous administration Alrp in intact rats induced a reduction of IFN- γ in liver-resident NK cells, while the administration of IFN- γ in 70% of partially hepatectomized rats was followed by a significant reduction of both mtTFA expression and liver regeneration. These results demonstrated a direct link between Alrp and IFN- γ , implying IFN- γ as the main mediator of Alrp biological activity both as growth factor and immunoregulator^[69]. Recent studies discussed a constructed yeast expressive vector of ALR and this was expressed in yeast cells in order to further study the biological function and mechanism of recombinant human ALR as a regulatory factor that specifically stimulates hepatic cell regeneration. This successful expression of ALR in yeast cells may pave the way for the study of clinical use and provided a good tool for further research in this field. Their results also suggested that ALR enhanced liver regeneration not only through an indirect pathway; i.e. immune regulation, but also through a direct pathway^[70-71].

Recently, an ALR recombinant plasmid was constructed and tested for therapeutic effects on rat hepatic fibrosis. Specifically, the results of this study revealed that the ALR recombinant plasmid could decrease the degrading capacity of collagen types I and III, which were regarded as important parameters reflecting the metabolism of collagen. The ALR recombinant plasmid could also decrease the deposition of the extracellular matrix and the expression of tissue inhibitors of metalloproteinase-1 (TIMP-1) in pathological liver tissue and thus reverse the hepatic fibrosis induced by porcine serum administration^[72].

In addition, the full-length mammalian Alrp (23 ku) was largely located in the mitochondrial intermembrane space and performed the export of iron/sulfur (Fe/S) clusters from the mitochondrial matrix, contributing to the biogenesis of cytosolic Fe/S proteins and to cellular iron homeostasis. Especially, Fe/S cluster components of the mitochondrial matrix preassembled and packaged Fe/S clusters, which could then be transported into the cytosol, a process possibly involving the ABC transporter Atm1p/ABC7^[45]. Cytosolic and nuclear Fe/S proteins have important regulatory functions as enzymes or transcription factors. Therefore, participation in the assembly of cytosolic Fe/S proteins appears to be the primary essential task of mitochondrial full-length Alrp^[45]. This function is crucial for all eukaryotic cell types, whereas the proposed role of 15 ku Alrp as a hepatotrophic factor is restricted to liver cells and it may be effective only after liver damage. This indicates that Alrp, like other redox-active proteins and sulfhydryl oxidases, may have diverse functions in the regulation of cell growth and differentiation^[20,48,57,60,65].

Klissenbauer *et al* first characterized full-length Alrp as intratesticular sulfhydryl oxidase, a new enzyme with expression regulated during spermatogenesis^[73]. Mitochondrial localization of full-length Alrp was of special interest for spermatogenesis because it was known that morphological and functional changes in mitochondria were associated with this highly complex cytodifferentiation process. They also observed the greatest amounts of this protein in spermatogonia and early spermatocytes. Expression levels of Alrp did not correlate with synthesis of components of the respiratory chain, indicating that the full-length Alrp in the mitochondria of spermatogonia and spermatocytes had another possible function in addition to its role in oxidative phosphorylation during sperm cell differentiation^[73].

CONCLUSION

ALR is novel growth factor, related to HSS, participating in the regulation of liver regeneration. With the availability and description of the ALR gene and gene product, a range of questions could be addressed about the cellular mechanisms in which this growth factor participates. At the molecular level, cloning of human ALR cDNA has been completed, but transcription and post-transcriptional regulation, based on the genomic structure of ALR, remains unclear. Transcription and post-transcriptional regulation are among the most important steps in the regulation of human gene expression. Therefore, it is important and essential to investigate the structure of human ALR genomic DNA. Contrary to previous belief, variable RNA expression in nonhepatic tissues suggests that ALR is not restricted to liver tissue, as was also suggested for HSS, but could be expressed in other cell types under appropriate circumstances. It is known that Alrp acts as a FAD-linked sulfhydryl oxidase belonging to the new Erv1p/Alrp family and is the only intratesticular sulfhydryl oxidase detected in the mitochondrial intermembrane space. Possible functions of sulfhydryl oxidases and other redox-active proteins in the regulation of cell growth, differentiation, changes in mitochondrial and cellular morphology, and in the formation of the

extracellular matrix have already been proposed. This indicates that Alrp, like other redox-active proteins and sulfhydryl oxidases, might have diverse functions. Additionally, limited data are available on the secreted Alrp bound to a specific receptor on the hepatocyte membrane and initiation of a signal transduction pathway and mediation of its biological effect on hepatocytes. Further studies should examine the distribution of human Alrp receptors on hepatocytes and on other cell types. The secretory pathway should also be further investigated because Alrp does not contain a typical signal peptide sequence evident of its primary structure. Moreover, Alrp production, expression regulation, secretion-transportation and importance, and also its genetic function, need to be further delineated. As a consequence, there is a necessity to extend studies on mammalian Alrp to different tissues, organs and developmental processes in conditions of normal and abnormal cellular growth. It is clear that the multiple functions of Alrp are biologically and physiologically complicated and need further study.

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Differential expression of apoptosis related proteins and nitric oxide synthases in Epstein Barr associated gastric carcinomas

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Abstract

AIM: To determine the incidence of Epstein Barr virus associated gastric carcinoma (GC) in Brazil and compare the expressions of apoptosis related proteins and nitric oxide synthases between EBV positive and negative gastric carcinoma.

METHODS: *In situ* hybridization of EBV-encoded small RNA-1 (EBER-1) and PCR was performed to identify the presence of EBV in GCs. Immunohistochemistry was used to identify expressions of bcl-2, bcl-xl, bak, bax, p53, NOS-1, NOS-2, and NOS-3 proteins in 25 EBV positive GCs and in 103 EBV negative GCs.

RESULTS: 12% of the cases of GC (25/208) showed EBER-1 and EBNA-1 expression. The cases were preferentially of diffuse type with intense lymphoid infiltrate in the stroma. EBV associated GCs showed higher expression of bcl-2 protein and lower expression of bak protein than in EBV negative GCs. Indeed, expressions of NOS-1 and NOS-3 were frequently observed in EBV associated GCs.

CONCLUSION: Our data suggest that EBV infection may protect tumor cells from apoptosis, giving them the capacity for permanent cell cycling and proliferation. In addition, EBV positive GCs show high expression of constitutive NOS that could influence tumor progression and aggressiveness.

INTRODUCTION

Gastric carcinoma (GC) is one of the most common malignant tumors in the world, although its incidence has gradually declined in recent years. In Brazil, gastric cancer remains a prevalent neoplasm with high mortality, ranked as the third highest cause of cancer-related deaths^[1].

The correlation between Epstein-Barr virus (EBV) infection and GC is well known. EBV infection is found in 2%-16% of GCs^[2-4]. However, the pathogenic role of EBV infection in GCs remains uncertain and little is known regarding the molecular characteristics of these tumors.

Apoptosis is a biological phenomenon of critical importance in the regulation of a number of physiological and pathological situations, such as cancer development. In general, it is thought that viral infection and its associated proteins protect against apoptosis, which would normally cause cancer regression^[5]. Among the genes that regulate apoptosis are the members of the bcl-2 family. Expression of bcl-2 protein in gastric tumor cells is frequent and may differ according the histological type^[6]. There are few studies that compare the relationship between EBV infection in gastric carcinomas with apoptosis related protein expression^[7-9].

Nitric oxide (NO) is a short-lived biomolecule with various biological functions. It is an important bioactive agent and signaling molecule that mediates a diverse array of actions such as vasodilatation, neurotransmission, and iron metabolism. NO is also involved in the multi-step process of carcinogenesis by mediating DNA damage, inducing angiogenesis, and suppressing immune responses^[10]. It has been demonstrated that low concentrations of NO can protect tumor cells against apoptosis^[11]; alternatively, high concentrations of NO

can induce tumor cell death^[12]. This small molecule is a product of the conversion of L-arginine to L-citrulline by nitric oxide synthases (NOS). There are two groups of NOS, inducible (iNOS or NOS-2) and constitutive (nNOS or NOS-1 and eNOS or NOS-3). Recent studies have examined the expression and activity of NOS in human cancer^[13,14]. In gastric carcinomas, many studies addressed the issue, and most of them showed an increased activity and expression of NOS in tumor tissue when compared with normal gastric mucosa^[15-18].

To the best of our acknowledgement, the relationship between NOS expression and EBV related gastric cancer has not been investigated. Thus, the aim of the present study is to determine the prevalence of EBV infection in GCs in a Brazilian population and investigate the expression of apoptosis related proteins and nitric oxide synthases in EBV positive and EBV negative gastric carcinomas.

MATERIALS AND METHODS

Materials

Two hundred and eight gastric carcinomas, surgically resected from 1995 to 1998, were analyzed for EBV status using in situ hybridization and PCR. To compare EBV positive and negative carcinomas, we selected 103 EBV negative GC, which were samples that were previously studied for expression of apoptosis related proteins and NOS. The cases were reviewed, and representative formalin-fixed, paraffin-embedded blocks from the tumors were selected. Clinicopathological findings were obtained from surgical records and pathology reports. New paraffin sections were stained with hematoxylin and eosin (H&E), and they were assessed independently by two pathologists (MDB & FAS). Histologically, the tumors were classified according to Lauren's classification system in intestinal or diffuse types^[19]. Also we graded the amount of lymphoid infiltrate within or between the tumor cells as minimal/mild or moderate/intense.

In situ Hybridization (ISH)

Sections (5-6 μm thick) on glass slides were prepared for ISH with a fluorescein-conjugated oligonucleotide probe for EBER-1 (Novocastra, Newcastle- Upon- Tyne, U.K.). Briefly, after deparaffinization and dehydration the sections were treated with proteinase K (Sigma, St. Louis, MO) for 15 min at 37°C. After washing and dehydration, the FITC-conjugated probe was applied and hybridized overnight at 37°C. The hybridization was further detected by rabbit anti- FITC antibody conjugated with alkaline phosphatase (Novocastra) for 30 min at room temperature. The slides were counterstained with a light Mayer's haematoxylin. Negative controls had no EBER-1 probe applied.

Immunohistochemistry

Sections (5-6 μm thick) from the same tumor blocks, used for EBER detection, were immunohistochemically analyzed using the standard streptavidin- biotin- peroxidase method. The staining was done using the microwave antigen retrieval technique (95°C for 30 min in citrate buffer pH 6.0)

for all antibodies. Sections were incubated with antibodies against p53 (DO-7, 1:100, Dako, Copenhagen, Denmark), Bcl-2 (124, 1:50, Dako, Copenhagen, Denmark), Bax (bax, 1:50, Dako, Copenhagen, Denmark), Bcl-xl (bcl-xl, 1:50, Dako, Copenhagen, Denmark), Bak (bak, 1:400, Dako, Copenhagen, Denmark), NOS-1 (nNOS, 1:200, Transduction Laboratories, USA), NOS-2 (iNOS, 1:40, Transduction Laboratories, USA), and NOS-3 (eNOS, 1:50, Transduction Laboratories, USA) at 4°C overnight. After reagenting them with biotinylated secondary anti-mouse antibodies, the antigen-antibody reactions were visualized using streptavidin-horseradish peroxidase conjugate (Dako LSAB Kit, Los Angeles, CA). The peroxidase activity was localized by 0.05% 3, 3'-diaminobenzidine and 0.03% hydrogen peroxide in Tris- buffered saline. The slides were counterstained with Mayer's hematoxylin. The percentage of positively stained tumor cells in each tumor section was blinded evaluated by counting at least 1000 cells in 10 randomly selected high-power fields. Brown staining for p53 protein was located in nuclei; staining for bcl-2, bcl-xl, bak, bax, NOS-1, NOS-2, and NOS-3 protein was located in cytoplasm. The section was considered as expressing the protein if cellular staining was $\geq 5\%$ ^[5].

PCR studies for EBV

The DNA of cancerous tissues from 25 patients with EBV positive gastric carcinomas was obtained from paraffin-embedded surgical blocks. The DNA was extracted by prolonged proteinase K digestion. Briefly, after deparaffinization with xylene and rehydration in ethanol, the tissue was resuspended in 300 μL of SDS-Proteinase K solution (1% SDS; 9 mmol/L Tris-HCl, pH 9.0; 2.25 mmol/L EDTA; 56.5 mmol/L NaCl; 1 $\mu\text{g}/\mu\text{L}$ Proteinase K (invitrogen) and incubated at 48°C for 48 h. Each 12 h, 20 μL of 20 $\mu\text{g}/\mu\text{L}$ Proteinase K were added. After extraction with an equal volume of phenol-chloroform, the aqueous layer was transferred to a new tube and the DNA was precipitated by the addition of 2 μL of 20 mg/mL glycogen, 100 μL of ammonium acetate (7.5 mol/L) and 800 μL of 100% ethanol followed by incubation overnight at -20°C. After centrifugation, the DNA was resuspended in 50 μL of water. PCR was performed in a reaction final volume of 25 μL , containing 10 μL of DNA; 2.5 μL of 10 \times PCR buffer; 1.5 μL of 25 mmol/L MgCl₂; 2 μL of 2.5 mmol/L dNTP mix; 1 μL of 10 $\mu\text{mol}/\text{L}$ EBNA1F and EBNA1R primers; and 0.3 μL of Taq DNA polymerase. The following thermal cycle conditions were used: 94°C for 5 min, 35 cycles of 94°C for 30 s, 60°C for 45 s, and 72°C for 45 s. After the last cycle, a final extension at 72°C for 7 min was carried out. The sequences of the primers were: EBNA-1F (5'-GTCATCATCATCCGGGTCTC-3') and EBNA-1R (5'-TTCGGGTTGGAACCTCCTTG-3'). The amplified products were visualized by electrophoresis in 8% polyacrylamide gels.

Statistical analysis

Statistical comparison among groups was performed using χ^2 test and Fisher's exact test when appropriate. *P* values less than 0.05 were considered statistically significant.

Table 1 Comparison of clinicopathological data between EBV positive and EBV negative gastric carcinomas

	EBV positive gastric carcinomas (n = 25)	EBV negative gastric carcinomas (n = 103)	P value
Gender			NS
Male	20 (80%)	68 (66%)	
Female	5 (20%)	35 (34%)	
Age: range(mean) ¹	35-78 (60.5)	25-84 (61.2)	NS
Site of tumor			NS
Body and Cardia	8 (32%)	21 (20%)	
Antrum	17 (68%)	82 (80%)	
LN metastasis			NS
Absent	7 (28%)	43 (41%)	
Present	18 (72%)	60 (59%)	
Histological type			< 0.05
Diffuse	18 (72%)	41 (40%)	
Intestinal	7 (28%)	62 (60%)	
Lymphoid stroma			< 0.05
Minimal/mild	6 (24%)	71 (69%)	
Moderate/intense	19 (76%)	32 (31%)	

¹ Age in years; LN: Lymph node; NS: Not significant.

RESULTS

EBER-1 expression and EBNA-1 products were visualized in 25 out of 208 (12%) cases of gastric carcinomas (Figure 1A).

The clinicopathological characteristics of EBV positive and negative GCs are given in Table 1. The male/female ratio in patients with EBV positive GCs and those with EBV negative GCs was 4:1 and 2:1, respectively, and no difference was seen in the average age at 60.5 and 61.2 years, respectively. 68% of EBV positive GCs were in the body and cardia and 32% of them were in the antrum. In the group of EBV negative GCs, 80% were in the body and cardia and only 20% of them were in the antrum, showing predilection for the body and cardia region in both groups. Regarding histological type, 18 of 25 (72%) EBV positive GCs were diffuse type and 7 (28%) were intestinal. 60% of EBV negative GCs were intestinal type and 41 of 103 (40%) were diffuse, resulting in a strong association between histological type and EBV infection in gastric carcinomas ($P < 0.01$). EBV associated GCs also correlated with tumor infiltrating lymphocytes. 19 of 25 (76%) EBV positive GCs showed marked lymphoid stroma evenly distributed with the malignant epithelial cells (Figure 1B). In the EBV negative GCs only 30% of the cases showed marked lymphocytic infiltration ($P < 0.01$). Metastases in lymph nodes were found in 18 of 25 (72%) EBV positive GCs and in 60 of 103 (59%) EBV negative cases. This difference was not statistically significant.

Immunohistochemical results

Table 2 shows the pattern of protein expression in EBV positive and negative GCs. P53 protein expression was observed in the nucleus of the tumor cells with a uniform staining (Figure 1C). 11 of 25 (44%) EBV positive GCs and 55 of 103 (51%) EBV negative GCs were positive for

Table 2 Apoptosis related proteins expressions in EBV positive and EBV negative gastric carcinomas n (%)

	EBV positive gastric carcinoma (n = 25)	EBV negative gastric carcinoma (n = 103)	P value
P53			NS
Positive	11 (44)	55 (54)	
Negative	14 (56)	48 (46)	
Bcl-2			< 0.05
Positive	7 (28)	11 (11)	
Negative	18 (72)	92 (89)	
Bcl-x			NS
Positive	22 (88)	96 (93)	
Negative	3 (12)	7 (7)	
Bak			< 0.05
Positive	11 (44)	71 (69)	
Negative	14 (56)	32 (31)	
Bax			NS
Positive	24 (96)	88 (86)	
Negative	1 (4)	15 (14)	

p53 and no difference was observed between the groups. Expression of bcl-2 protein was observed in 28% of EBV positive GCs and in only 11% of the EBV negative GCs (Figure 1D), showing a statistically significant correlation between EBV status and bcl-2 expression in GCs ($P < 0.05$). The rates of the bcl-xl and bax expressions in EBV positive and negative GCs were 88% and 93%, and 96% and 86%, respectively (Figures 1E and 1F). None of the differences were statistically significant. Bak expression was lower in the EBV positive GCs than in EBV negative tumors (Figure 1G) and it was detected in 11 of 25 (44%) EBV positive GCs, and in 71 of 103 (69%) EBV negative GCs. This difference was statistically significant ($P < 0.01$).

NOS were observed in the cytoplasm of the tumor, epithelial, endothelial, smooth muscle and inflammatory cells. For final analysis, we scored the expression of these enzymes only in the tumor cells (Figures 1H, 1I, and 1J). NOS expressions were significantly different in EBV positive and negative GCs. Overexpression of constitutive forms of NOS (NOS-1 and NOS-3) were more frequently observed in EBV positive than in EBV negative GCs ($P < 0.01$). The majority of GC cases were negative for an inducible form of NOS (NOS-2), independently of EBV status. Comparisons of NOS expressions and EBV status are shown in Table 3.

DISCUSSION

The relationship between Epstein-Barr virus and various epithelioid diseases has already been demonstrated. Involvement of EBV has been described in the etiopathogenesis of not only the nasopharyngeal carcinoma but other carcinomas as well, including gastric carcinomas^[20-22].

It is estimated that EBV infection can be found in about 10% of GCs worldwide, especially those with marked lymphocytic infiltration^[23-27]. We observed EBV infection in 12% of GCs. Previous studies showed the

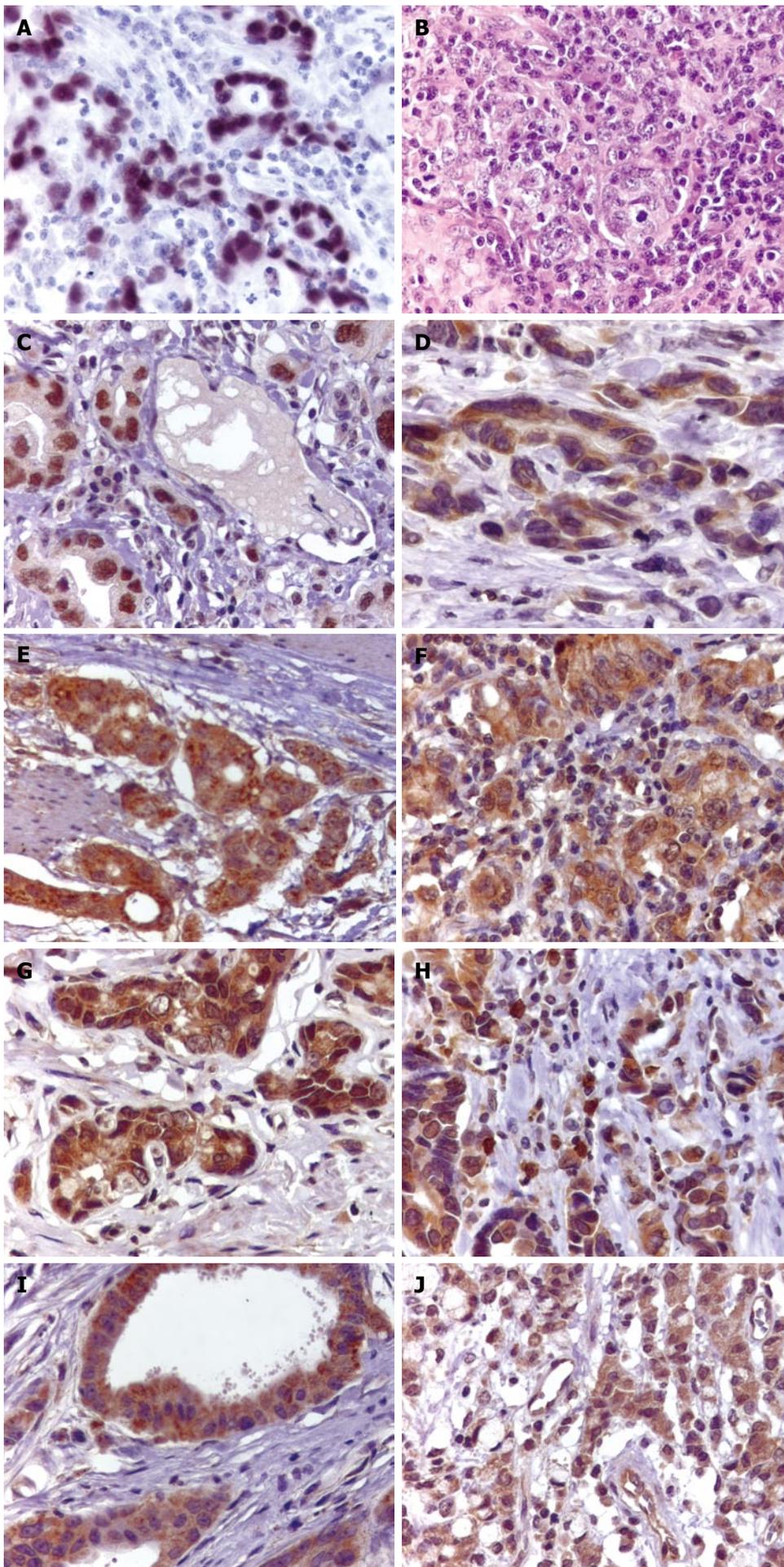


Figure 1 A: Gastric carcinoma showing strong positivity of EBER-1 in nuclei of the malignant cells (*in situ* hybridization,400 x); B: Overview of gastric carcinoma with clusters of tumor cells separated by prominent lymphoplasmacytic infiltrate (H&E, 400 x); C: p53 nuclear immunopositivity in gastric carcinoma cells (400 x); D, E, F, G: Immunohistochemical staining for bcl-2, bcl-xl, bax and bak. The cytoplasm of the malignant cells shows irregular brown positivity (400 x); H, I, J: NOS-1, NOS-2 and NOS-3 immunohistochemical staining in tumor epithelial cells, showing a high intensity of staining in the cytoplasm (400 x).

incidence of EBV infection in Brazilian GCs ranging between 5%-11.32%^[28-30]. Some reports described the presence of EBV and its products as restricted on the carcinoma cells^[31-33], whereas others found this virus in the precursor epithelial lesions as well as in the lymphoid cells^[2,7]. In our study the virus was detected in the neoplastic cells but not in the normal, dysplastic gastric epithelium, or in inflammatory cells. This finding indicates that EBV affects the gastric epithelium at a late stage of the multistep process of gastric carcinogenesis.

The role of EBV in neoplastic transformation of gastric epithelial cells is not completely understood. It has been suggested that EBV positive GCs display specific clinicopathological features compared with EBV negative GCs. EBV associated GCs had been characterized by male predominance, preferential location in proximal stomach, and a high prevalence of diffuse types^[2,34-36]. In our series, EBV positive GCs were frequently seen in males, in the body and cardia region, and in diffuse carcinomas in agreement with previous reports. Indeed we observed a strong association between EBV infection and lymphoid stroma. The presence of marked lymphoid infiltrate has already been demonstrated and there is growing evidence that extensive lymphocyte infiltration is a consistent characteristic of EBV positive carcinomas and correlates with less aggressive behavior, although no additional information is available regarding follow-up of the patients in the present study.

Molecular analysis of EBV positive carcinoma has been shown to have distinct characteristics including different chromosomal aberrations^[37], different and lower frequencies of microsatellite instability^[35] and allelic loss^[38], and more CpG methylation^[39]. However, studies have focused on the relationship between EBV and oncogenes or tumor suppressor genes in EBV associated GCs, but no conclusive results have been reported^[5,7,9,40,41]. In this study, expression of apoptosis related proteins and nitric oxide synthases was examined in EBV associated and EBV negative gastric carcinomas. In this report, we observed a correlation between bcl-2 and bak expressions and EBV status. Bcl-2 protein expression was observed more frequently in EBV positive than in EBV negative GCs. On the other hand, positivity for bak protein was more common in EBV negative than in EBV positive GCs. It is already known that the balance between anti-apoptotic and pro-apoptotic members of the bcl-2 family genes determine the outcome of patients with tumors. Bak protein is shown to induce cell death, while bcl-2 can protect cells from apoptosis. Previous reports indicated that EBV infection may protect tumor cells from apoptosis inducing expression of the anti-apoptotic proteins^[41]. Our results showed additional information and confirm that EBV infection and apoptosis related proteins interact to negatively influence apoptosis, through high expression of bcl-2 and low expression of bak in gastric tumor cells. Bax and bcl-xl protein expressions were found in both EBV positive and negative GCs and there was no statistical difference between the groups.

There is some indication that EBV modulates and mutates p53 for its own survival^[42], however earlier reports showed contradictory results^[8,38,43]. In turn, p53

Table 3 NOS expression in EBV positive and EBV negative gastric carcinomas *n* (%)

	EBV positive gastric carcinoma (<i>n</i> = 25)	EBV negative gastric carcinoma (<i>n</i> = 103)	<i>P</i> value
<i>NOS1</i> (<i>nNOS</i>)			< 0.05
Positive	23 (92)	69 (67)	
Negative	2 (8)	34 (33)	
<i>NOS2</i> (<i>iNOS</i>)			NS
Positive	5 (20)	31(30)	
Negative	20 (80)	72 (70)	
<i>NOS3</i> (<i>eNOS</i>)			< 0.05
Positive	18 (72)	36 (35)	
Negative	7 (28)	67 (65)	

gene mutations are rarely identified in EBV associated carcinomas^[44]. The obtained results in our study showed disturbed function of p53 in almost all cases of EBV positive and EBV negative GCs. Although p53 expression seemed to be slightly lower in EBV positive GCs, that difference was not statistically significant. These results indicated that abnormalities in p53 observed in GCs are independent of EBV infection.

It has been reported that nitric oxide synthases are present in human tumor cell lines and solid tumor tissues^[14,16,45-47]. Increased NOS expression has been observed in different tumors tissues when compared with normal tissues^[14,48,49].

To the best of our knowledge, this is the first report showing NOS expression is associated with EBV infection gastric cancer. In EBV positive GCs, NOS-1 and NOS-3 proteins were more frequent positive in tumor cells than NOS-2. The results agree with previous data that showed higher activity of constitutive rather than inducible NOS in human gastric tumors^[15]. In a recent study, the authors demonstrated significantly high NOS-3 expression in gastric tumors and a directly relationship with tumor angiogenesis and the overall aggressive biology of gastric cancer^[50]. Our study showed higher NOS-3 expression in EBV positive GCs than in EBV negative GCs. Increasing evidence suggests that NOS-3 expression, although constitutive, can be regulated by various hormones, cytokines, and growth factors, and by genetic alterations, such as oncogene activation and tumor suppressor inactivation^[51-54]. Our data indicate that EBV infection and its products can affect the regulation of this enzyme resulting in elevated NO production. It has been showed that elevated NO production enhances the growth of some tumors through the suppression of anti-tumor immune responses^[55-57].

NOS-2 expression was decreased in the whole group of gastric tumors in our study. This feature has been demonstrated by immunohistochemistry in other tumors and there is a possible relationship between loss of NO and carcinogenesis^[15,16]. However, many publications showed elevated NOS expression and increased activity in gastric cancer^[58-60]. Although the mechanisms for the antiviral action of NO have not been clarified, the multiplicity of host enzymes it targets makes it possible

that multiple alterations in host cell proteins are involved. An apparent role of NO in inhibiting EBV reactivation from latency was demonstrated in a study using gastric cells lines in culture^[61]. This finding led us to propose that EBV latency can be directly and/or indirectly associated with NOS gene expression and also lead us to postulate that NO might also be an important factor in the host EBV relationship. Our data suggest that the increased expression of NOS-1 and NOS-3 in EBV positive GC renders a major contribution to high activity of NOS in this specific group of tumors.

In conclusion, it is clear that EBV is associated with gastric carcinomas. EBV positive GCs have a distinct protein expression profile, as well as distinct clinicopathological features. This present data suggest that EBV infection may protect tumor cells from apoptosis, giving them the capacity for permanent cell cycling and proliferation. Indeed, EBV positive GCs have high expression of constitutive NOS, which might be associated with tumor progression or aggressiveness. A larger number of cases and long-term follow-up are necessary to further investigate this possibility.

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LIVER CANCER

Dynamic alteration of telomerase expression and its diagnostic significance in liver or peripheral blood for hepatocellular carcinoma

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Abstract

AIM: To investigate the dynamic alteration of telomerase expression during development of hepatocellular carcinoma (HCC) and its diagnostic implications in liver tissues or peripheral blood mononuclear cells for HCC.

METHODS: Dynamic expressions of liver telomerase during malignant transformation of hepatocytes were observed in Sprague-Dawley (SD) rats fed with 0.05% of 2-fluoenyacetamide (2-FAA). Total RNA and telomerase were extracted from rat or human liver tissues. The telomerase activities in livers and in circulating blood were detected by a telomeric repeat amplification protocol-enzyme-linked immunosorbent assay (TRAP-ELISA), and its diagnostic value was investigated in patients with benign or malignant liver diseases.

RESULTS: The hepatoma model displayed the dynamic expression of hepatic telomerase during HCC development. The telomerase activities were consistent with liver total RNA levels ($r = 0.83$, $P < 0.01$) at the stages of degeneration, precancerosis, and cancerization of hepatocytes. In HCC patients, the telomerase levels in HCC tissues were significantly higher than in their adjacent non-cancerous tissues, but liver total RNA levels were lower in the former than in the latter. Although the circulating telomerase of HCC patients was abnormally

expressed among patients with chronic liver diseases, the telomerase activity was a non-specific marker for HCC diagnosis, because the incidence was 15.7% in normal control, 25% in chronic hepatitis, 45.9% in liver cirrhosis, and 85.2% in HCC, respectively when absorbance value of telomerase activity was more than 0.2. If the value was over 0.6, the incidence was 60% in HCC group and 0% in any of the others ($P < 0.01$) except in two cases with liver cirrhosis. However, the combination of circulating telomerase with serum alpha-fetoprotein level could increase the positive rate and the accuracy (92.6%, 125 of 135) of HCC diagnosis.

CONCLUSION: The overexpression of telomerase is associated with HCC development, and its abnormality in liver tissues or in peripheral blood could be a useful marker for diagnosis and prognosis of HCC.

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Key words: Hepatocellular carcinoma; Telomerase; Peripheral blood mononuclear cells; Telomeric repeat amplification protocol

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common and rapidly fatal malignancies worldwide^[1], and has been ranked as the 2nd cancer killer in China, particularly in the eastern and southern areas, including the inshore area of the Yangtze River^[2]. Major risk factors for HCC in these areas are exposure to aflatoxin B1 (AFB1) and infection with hepatitis viruses (HBV and HCV)^[3]. Its prognosis is poor and early diagnosis is of utmost importance^[4]. Treatment options are severely limited by the frequent presence of metastases. Although serum alpha-fetoprotein (AFP) is a useful tumor marker for the detection and monitoring of HCC development, the false-negative rate with AFP level alone may be as high as 40%

for patients with small size HCC. If hepatocyte-specific mRNAs are detected in the circulation, it is possible to infer the presence of circulating, presumably malignant liver cells and to predict the likelihood of haematogenous metastasis^[5-7]. The use of telomerase assay for cancer diagnosis might be possible because telomerase is expressed in malignant lesions but rarely in normal somatic cells^[8].

Telomerase, an RNA-dependent DNA polymerase, can maintain the telomeric length by acting as a reverse transcriptase^[9]. In humans, tumor cells escape programmed cell senescence through reactivation of telomerase^[10]. These immortalized cells can compensate for telomeric shortening at each cell division, leading to progressive neoplastic evolution^[11]. Telomerase re-expression is found in 85% of human malignant tumors^[12]. The expression of telomerase is important to cell proliferation, senescence, immortalization and carcinogenesis^[13]. However, early expression and kinetic alteration of hepatic telomerase during development of HCC remain unclear. The objectives of this study were to observe the dynamic expression of liver telomerase in rat hepatoma model induced by oral administration of 0.05% of 2-fluorenylacetamide (2-FAA), and investigate the telomerase activities in liver tissues and peripheral blood mononuclear cells (PBMCs) by a telomeric repeat amplification protocol-enzyme-linked immunosorbent assay (TRAP-ELISA), as well as its diagnostic implications for HCC in patients with benign or malignant liver diseases.

MATERIALS AND METHODS

Hepatoma rat models

Forty-two closed colony male Sprague-Dawley (SD) rats weighing 120-160 g were obtained from the Experimental Center of Medical Animals, Nantong University, China, and divided randomly into 7 groups, one group ($n = 6$) as normal control and 6 experimental groups ($n = 6$ in each group). All rats were housed under bio-clean conditions at a temperature-controlled ($22^{\circ}\text{C} \pm 2^{\circ}\text{C}$) environment with a 12-h light/dark cycle and 55% humidity. The control group was given basal foodstuffs and the feed of experimental rats contained 0.05% 2-FAA (Sigma, USA). Rats were then monitored daily of survival and weight loss, recorded of their clinical signs and sacrificed at different stages of hepatoma development. One group of the experimental rats and one control rat were killed in every two weeks. All surgical procedures were conducted under deep ether anaesthesia. Blood was drawn from rat's heart. The livers were collected, and histological examination was performed with hematoxylin and eosin (HE) staining. All procedures performed on the rats were conducted in accordance with the Guidelines for Experimental Animals approved by the Animal Care and Use Committee of Nantong University, China.

Patient selection and human HCC tissues

All patients with liver diseases came from the Affiliated Hospital, Nantong University, China. We evaluated 135 HCC patients (103 males and 32 females, age between 35-76 years, mean 49). Ninety-five patients (70.3%)

had a history of cirrhosis, and 52 (38.5%) had a history of chronic hepatitis. Moreover, 85.2% (115/135) were hepatitis B surface antigen (HBsAg) carriers, 10.4% (14/135) had antibodies to hepatitis C virus (anti-HCV, second-generation antibody) and 8.9% (12/135) had antibodies to hepatitis G virus by an enzyme-linked immunosorbent assay (ELISA), respectively. Other cases studied included 80 patients with chronic hepatitis (CH, 58 males and 22 females), 37 patients with liver cirrhosis (LC, 30 males and 7 females), and 70 healthy individuals (35 males and 35 females, mean age 35 years) with negative hepatitis B virus markers (HBsAg, HBcAb, and HBV-DNA) and anti-HCV, and normal levels of serum alanine aminotransferase (ALT) from the Nantong Central Blood Bank as normal controls (NC). The venous blood of patients and normal controls were collected with heparin anticoagulant and stored on ice. The diagnosis of HCC and viral hepatitis was based on the criteria proposed at China National Collaborative Cancer Research Group^[14] and the China National Viral Hepatitis Meeting^[15], respectively.

Fifteen specimens of HCC and their corresponding non-tumor tissues as self-control were obtained from HCC patients who had undergone curative hepatectomy. These specimens were immediately frozen in liquid nitrogen and kept at -85°C . All specimens were hepatocellular cancer confirmed by pathological examination. Eleven non-cancerous tissues from those patients were atypical proliferation. The patients' ages ranged between 31-64 years (mean 51.3 years). There were 12 specimens with a tumor size larger than 3 cm, and 3 specimens smaller than 3 cm.

Isolation of total RNA

Fifty mg of each liver tissue (rat liver, human HCC, and their self-controlled non-tumor tissues) was homogenized with a Polytron homogenizer after the addition of 1.0 mL of TRIzol reagent (Promega). Then 0.2 mL of chloroform was added into the tubes, mixed by vortexing for 15 s, and placed at -20°C for 5 min. Samples were centrifuged at 12000 r/min for 15 min at 4°C . The supernatants were collected, and equivalent isopropanol was added and then they were put into another tube and mixed gently, placed at -20°C for 15 min, then centrifuged at 12000 r/min for 15 min at 4°C . The supernatants were removed, the RNA pellets were washed twice with 0.5 mL of 75% ethanol, mixed and centrifuged at 8000 r/min for 8 min at 4°C . The RNA pellets were air dried 5 min at room temperature and reconstituted in 20 μL of RNase-free DEPC water and incubated at 60°C for 10 min. The purity and concentration of the RNA was estimated by the ratio of absorbance readings at 260 and 280 nm, with an $A_{260/280}$ ratio between 1.8 and 2.0 indicating sufficient purity. RNA samples were kept frozen at -85°C until use.

Separation of PBMCs

Five milliliters of peripheral blood were collected with heparin anticoagulant from each patient. Ficoll (2.5 mL) was added to each sample. After centrifugation at 2000 r/min for 20 min, the PBMCs were collected from the Ficoll/plasma interface. Then they were washed three times in normal saline and pelleted by using low-speed cen-

trifugation. The cells were collected at 2×10^5 /tube, then stored at -85°C for telomerase assays.

TRAP reaction

A commercial telomerase TRAP-ELISA kit (Boehringer Mannheim), based on TRAP introduced by previous methods^[16], was used to assay telomerase activity in all specimens. Fifty mg liver tissue or 2×10^5 cells were suspended in 200 μL lysis reagent and incubated for 30 min, then centrifuged at 16000 r/min for 20 min at 4°C . Positive control cell extract was from immortalized telomerase-expressing human kidney cells (293 cells). Negative control cell extract was as follows: heat-treatment of the cell extract for 10 min at 65°C prior to the TRAP reaction. The supernatant was removed to other EP tubes and kept frozen at -85°C . Telomeric repeats were added to a biotin-labeled primer for 30 min at 25°C during the first reaction. The mixture was incubated at 94°C for 5 min to induce telomerase inactivation, then subjected to 30 PCR cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 90 s, and finally at 72°C for 10 min. The PCR products were used for analysis of telomerase activity and gel electrophoresis.

Telomerase detection

An aliquot of the PCR product was denatured and hybridized to a digoxigenin-(DIG)-labeled, telomeric repeat-specific detection probe. The resulting product was immobilized *via* the biotin labeled primer to a streptavidin-coated microtiter plate. The detection probe and the hybridization conditions have been optimized to obtain the highest specificity and sensitivity. The immobilized PCR products were then detected with an antibody against digoxigenin (anti-DIG-POD) that was conjugated to peroxidase. Finally, the probe was visualized by use of peroxidase metabolizing tetramethyl benzidine (TMB) to form a colored reaction product. The absorbance (A) of the samples was measured at 450 nm (reference wavelength 620 nm) within 30 min of addition of the stop reagent. Telomerase activities were expressed according to absorbance difference (ΔA) between sample and negative control.

Telomerase by TRAP silver staining

Fifteen microliters of the PCR products were loaded to 12% polyacrylamide gel and resolved by electrophoresis at 160-180 V for 1-2 h. Then the gel was immobilized to 10% ethanol for 5 min, soaked in 1% AgNO_3 for 5 min, dyed in silver dye solution for 10 min, and then in developing dye for 10 min. The amplified products were of heterogeneous length and created a ladder pattern of bands each representing the addition of a hexanucleotide telomeric repeat by telomerase, and photographed by Delphin-Doc Digital Imaging System (Wealtec, USA). All samples were done in duplicate and the reproducibility was confirmed.

Statistical analysis

The livers of experimental rats according to pathological examination were divided into 4 groups: degeneration of hepatocytes (Exp-1), precancerosis of hepatocytes (Exp-2), cancerization of hepatocytes (Exp-3), and normal controls (Con). The telomerase activities in

Table 1 Histopathological alterations of hepatocytes at different stages of rat hepatomas induced with 2-FAA

Group	n	Pathological feature (HE staining)		
		Degeneration	Precancerosis	Cancerization
Control	6	0	0	0
2 nd wk	6	6	0	0
4 th wk	6	3	3	0
6 th wk	6	0	5	1
8 th wk	6	0	4	2
10 th wk	6	0	1	5
12 th wk	5 ¹	0	0	5
Total	41	9	13	13

¹ One rat died during the experimental process; 2-FAA: 2-fluoenylacetamide.

PBMCs were investigated in patients with HCC, chronic hepatitis (CH), liver cirrhosis (LC), and normal controls (NC). Human HCC tissues were divided into cancerous and their non-tumor (non-HCC) groups. Levels of telomerase and total RNA were expressed as mean \pm SD. Differences between groups were assessed by the Student's *t* test or the χ^2 test, and the Fisher's exact probability test. Probability values less than 0.05 were considered significant.

RESULTS

Histopathological findings

The pathological changes of experimental rat livers with HE staining are shown in Table 1. The rats were administered with 0.05% of 2-FAA for two weeks. The appearance of livers turned to be grey-yellow and scabrous. The hepatocytes showed granular degeneration. Most of normal liver tissue structures remained. A few of hyperplastic oval cells, and the tendency toward nodule formation were seen at the early stage of hepatoma development. At the end of four weeks, precancerous lesions appeared in half of liver tissues. Histologically, normal liver follicles existed in most areas but hyperplastic small round cells or oval cells or hyperplastic nodules were found locally. After the 6th wk, all livers progressed into canceration stage. The structures of liver lobules were completely destroyed, and necrosis was spread over the whole livers. Large numbers of small oval cells, and cancer nests were observed. The histological features of livers showed diffuse patchy necrosis and high degree differentiation of HCC.

Expression of telomerase during rat hepatoma development

The dynamic expression of liver telomerase in rat hepatocyte degeneration (Exp-1), hepatocyte precancerosis (Exp-2), and hepatocyte cancerization (Exp-3) groups during development of hepatomas in comparison with controls (Con) are shown in Figure 1. After rats were given 2-FAA, the specific activities ($\Delta A/\text{mg}$ protein) of liver telomerase were significantly higher than that of controls ($P < 0.01$) at the stage of hepatocyte degeneration, kept rising at the stage of hepatocyte precancerosis, and peaked at the formation stage of hepatoma. Telomerase activities were consistent with the levels of liver total RNA ($r = 0.83$,

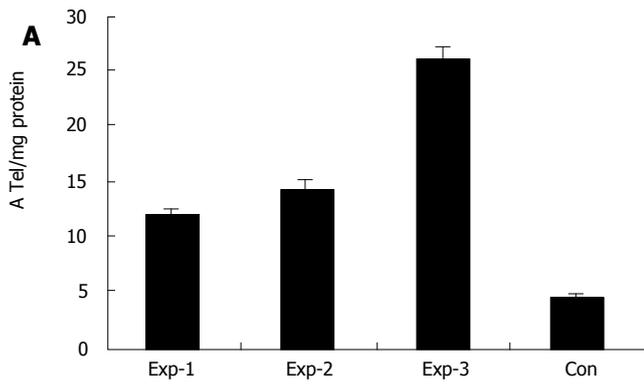


Figure 1 Alterations of hepatic telomerase at different stages of rat hepatoma model. The feed of experimental rats contained 0.05% of 2-fluoenylacetamide. Exp-1: Hepatocyte degeneration group; Exp-2: Hepatocyte precancerosis group; Exp-3: Hepatocyte cancerization group; and Con, rats given basal feed without 2-fluoenylacetamide as normal control group.

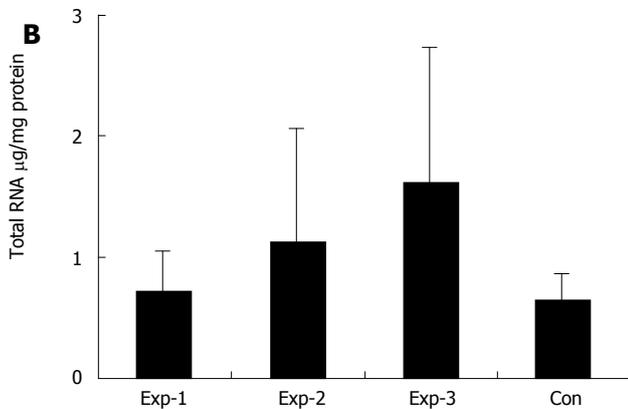


Figure 2 Alterations of liver total RNA at different stages of rat hepatoma model. The feed of experimental rats contained 0.05% of 2-fluoenylacetamide. Exp-1: Hepatocyte degeneration group; Exp-2: Hepatocyte precancerosis group; Exp-3: Hepatocyte cancerization group; and Con, rats given basal feed without 2-fluoenylacetamide as normal control group.

$P < 0.01$) at the different stages of hepatoma development of rats (Figure 2).

Expression of telomerase in human liver tissues

The levels of total RNA and telomerase expression in different human liver tissues are shown in Figure 3. The concentrations of liver total RNA and telomerase were $12.40 \pm 7.34 \mu\text{g/mg}$ liver protein and $18.25 \pm 15.02 \text{ A}/\mu\text{g RNA}$ in HCC tissues, and $53.77 \pm 52.02 \mu\text{g/mg}$ liver protein and $8.16 \pm 6.22 \text{ A}/\mu\text{g RNA}$ in their non-cancerous tissues, respectively. The expression of total RNA in HCC tissues was significantly lower than that in their non-cancerous tissues ($P < 0.01$); however, the expression level of telomerase was significantly higher in HCC tissues than in their non-cancerous tissues ($P < 0.05$). Stronger positive expression of telomerase was presented in HCC tissues and not in their non-cancerous tissues (Figure 4).

Significance of telomerase in PBMCs

There were different telomerase activities of PBMCs in NC, CH, LC and HCC, but the activities in HCC were significantly higher than in others ($P < 0.01$, Figure 5). The

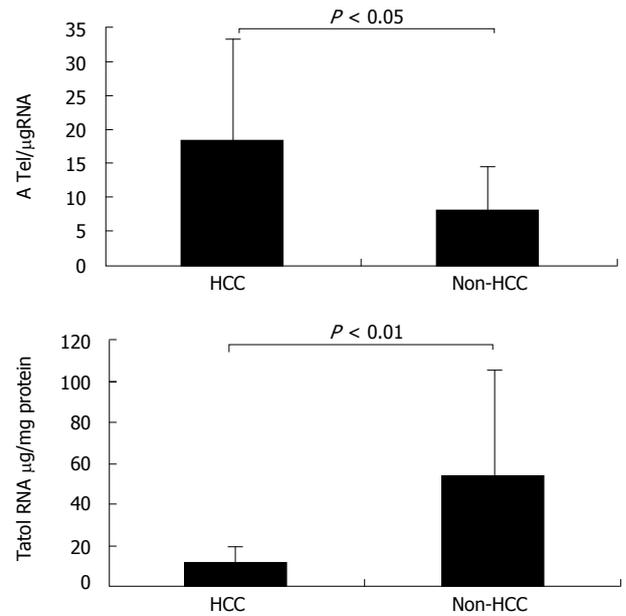


Figure 3 Comparative analysis of total RNA levels and telomerase activities in human hepatoma tissues and their non-cancerous tissues.

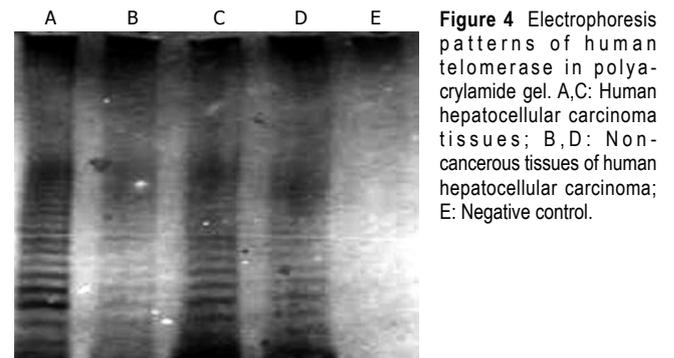


Figure 4 Electrophoresis patterns of human telomerase in polyacrylamide gel. A,C: Human hepatocellular carcinoma tissues; B,D: Non-cancerous tissues of human hepatocellular carcinoma; E: Negative control.

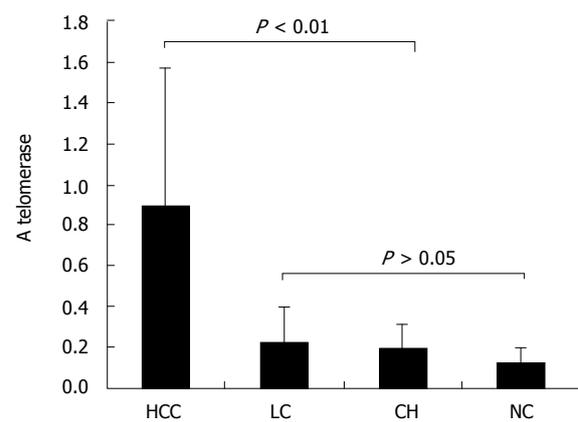


Figure 5 Comparative analyses of telomerase activities in peripheral blood mononuclear cells from patients with benign or malignant liver diseases.

samples were regarded as telomerase-positive if the difference in absorbance (Table 2) was greater than $0.2 \Delta\text{A}$. The incidence of telomerase expression was 15.7% in NC, 25% in CH, 45.9% in LC, and 85.0% in HCC (sensitivity 68.4% and positive predictive value 75.7%), respectively. The incidence of telomerase was significantly higher in

Table 2 Diagnostic values of different telomerase levels in peripheral blood mononuclear cells for hepatocellular carcinoma

Group	n	Positive (%)		
		$\Delta A > 0.2$	$\Delta A > 0.4$	$\Delta A > 0.6$
HCC	135	115 (85.2)	101 (74.8)	81 (60.0)
Liver cirrhosis	37	17 (45.9)	6 (16.2) ^b	2 (5.4) ^b
Chronic hepatitis	80	20 (25.0) ^b	8 (10) ^b	0 (0) ^b
Normal control	70	11 (15.7) ^b	0 (0) ^b	0 (0) ^b

^b*P* < 0.01 vs the HCC group.

Table 3 Assessment of the diagnostic values of different telomerase levels

Telomerase	Sen (%)	Spe (%)	Predictive value		
			Pos (%)	Neg (%)	Acc (%)
$\Delta A > 0.2$	85.2	68.4	75.7	80.0	77.4
$\Delta A > 0.4$	74.8	88.0	87.8	75.2	81.0
$\Delta A > 0.6$	60.0	98.3	97.6	68.0	77.8

Sen: Sensitivity; Spe: Specificity; Pos: Positive; Neg: Negative; Acc: Accuracy.

HCC group than in any of other groups (*P* < 0.01). If the value rose up to 0.6 ΔA , the incidence of telomerase was 60% in HCC (sensitivity 98.3% and positive predictive value 97.6%), and 0% in any of other groups (*P* < 0.01) except two cases with liver cirrhosis (Table 3). For combined diagnostic values of telomerase and AFP for HCC, the AFP concentration in HCC patients was 1765 ± 1705 $\mu\text{g/L}$ and the positive rate was 70% for a cutoff value of AFP level 200 $\mu\text{g/L}$. Total AFP detection in combination with telomerase activity could increase the accuracy of HCC diagnosis (92.6%).

DISCUSSION

Carcinogenesis of hepatocytes is a multi-factor, multi-step and complex process and has many characteristics, such as fast infiltrative growth, metastasis in early stage, high malignancy, and poor therapeutic efficacy^[17]. Probably HBV infection is merely a carcinogenic factor, and is not related to the growth, infiltration and metastasis of HCC^[18]. It is increasingly clear that oncogenesis is driven by the activation of telomerase, a ribonucleoprotein complex that adds telomeric repeats (hexanucleotide 5'-TTAGGG-3') to the ends of replicating chromosomes. Telomerase is a ribonucleoprotein composed of an essential RNA and a few proteins. It is expressed in embryonic cells and in adult male germ line cells, but is undetectable in normal somatic cells except proliferative cells of renewable tissues. Although the precise processes leading to HCC are still unknown, progression to a cancerous state does require the accumulation of a series of genetic alterations.

To understand the mechanisms and alterations of telomerase expression during carcinogenesis of hepatocytes, rat hepatoma models were induced with chemical carcinogen (2-FAA), and telomerase expression was studied at the different stages of degeneration, precancerosis, and cancerization of hepatocytes (Table 1).

Although the histopathological examination found the oval cells and non-differentiation in morphology at the early stage of hepatoma, telomerase was significantly expressed in livers (Figure 1). This has raised two possibilities: (1) telomerase may be an important target for therapy aiming at controlling cell growth^[19,20]. Telomerase activation in the critical step of tumor progression during hepatocarcinogenesis might be regulated only by hTERT, whereas increased telomerase activity in tumor progression might be regulated by both hTERT (reverse transcript) and hTERT (RNA component)^[21-23]; (2) histological examination in combination with telomerase activity could increase the accuracy of diagnosis at early stage and help the staging of HCC development.

The predominant mechanism for telomere stabilization in tumor cells is the activation of the telomere-synthesizing enzyme telomerase. Both of liver telomerase activity (Figure 1) and total RNA increased (Figure 2) during hepatoma progression. This may be relevant to the cytotoxic effects of liver cells in resisting carcinogenic effect of 2-FAA. In the meantime, the telomerase level of liver tissues and sera of the experimental groups were obviously higher than the normal control group (*P* < 0.01), indicating that the oval cells could express telomerase and secrete it into blood. Telomerase activities increased continuously and reached the highest level in the experimental groups, and livers of the experimental groups in the 6th wk were seen to have canceration. Rat hepatoma is an aggressive malignancy. Both total RNAs and telomerase activity increased during HCC progression. Moreover, in the following weeks, the liver tissues continued to synthesize telomerase, which remained at a high level. These results suggest that the strong enhancement of telomerase activity may be a critical step in hepatocarcinogenesis, although the exact mechanism is not clear.

Telomerase is highly activated in human tumor tissues, whereas it is not activated in primary cell strains and tumor-adjacent tissues^[24,25]. With cell division, telomerase is activated. The cells with positive telomerase activity become immortalized and further develop to carcinoma cells^[26,27]. The telomerase activities in HCC and their surrounding non-tumor tissues were detected by TRAP-ELISA in this study. The telomerase expression in HCC was negatively correlated with differentiation, but was not related to tumor size or histological grade. The expression level of telomerase was significantly higher in HCC tissues than in their corresponding non-cancerous tissues (*P* < 0.05, Figure 3A). Stronger positive expression of telomerase was present in HCC and not in their non-cancerous tissues (Figure 4). No relationship could be observed between the enhancement of telomerase activity and liver total RNA in human HCC tissues (Figure 3B). In China, major risk factors for HCC are exposure to aflatoxin B1 (AFB1) and infection by hepatitis viruses, especially HBV. HBx, encoded by HBV, could block the function of *p53*, resulting in genome unstabilization. Activation of telomerase could make liver cells immortalized, and microvascularization could provide nutrition for their growth and promote metastasis^[17,28]. These two factors may induce carcinogenesis and have a key role in occurrence, development, infiltration and metastasis of HCC. They

may also have notable clinical values in diagnosing and treating HCC in early stage.

Telomerase was strongly activated in HCC, while weakly activated in CH and LC. Progressive shortening of telomeres during hepatocellular carcinogenesis was found from normal liver to CH to LC to HCC. The average telomere length of HCC was significantly and consistently shorter than that of adjacent CH or LC. The possible sequel of telomere shortening in regenerative, non-cancerous liver lesions (CH and LC) is that it may eventually lead to reactivation of telomerase, which in turn may contribute to the malignant conversion to HCC. Telomerase may be expressed in lymphocytes^[29,30]. Lymphocytic infiltration may occur during tumor necrosis, thus the telomerase activity we measured included a proportion generated by lymphocytes and might be overestimated. Almost all HCCs are preceded by CH and/or LC. There were different degrees of telomerase activities of PBMCs in NC, CH, LC and HCC, but the telomerase activities of HCC were significantly higher than others (Figure 5, Table 2). In addition, combined diagnostic values of telomerase and AFP for HCC were investigated in this study. Detection of telomerase activity in combination with serum AFP could increase the accuracy of HCC diagnosis.

These genetic changes may lead to senescence of hepatocytes occurring in patients with advanced liver diseases probably as a result of degeneration and the subsequent regeneration of hepatocytes^[31,32]. Although the mechanisms of carcinogenesis in chronic liver diseases are not known, recessive mutations may result in the reactivation of telomerase and stabilization of telomere length, as well as fixation of the additional mutations required for invasiveness and metastasis^[33,34]. Moreover, the patients with telomerase-positive HCC show poorer prognosis than those with telomerase-negative tumors. Clinically although the diagnostic specificity of hepatoma is high, the positive rate of telomerase is at present much lower than that from animal model studies, and the number of bands does not coincide with that of human being either^[35]. In addition, the cause of human hepatoma is associated with many factors. Telomerase could become a valuable marker for HCC diagnosis if the sensitivity of detection method is improved^[36]. Further research should focus on mechanisms of carcinogenesis by HCV and HBV, development of early detection and more effective treatments. Better understanding of HCC related to hepatitis viruses should also be a major concern in future studies.

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Analysis of differentially expressed proteins in cancerous and normal colonic tissues

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INTRODUCTION

Colorectal cancer is a worldwide public health concern and also a frequent cause of mortality and morbidity in developed, developing and industrialized countries^[1]. The incidence of colon cancer increases exponentially with age. Environmental factors and host immunological characteristics could contribute to initiation and progression of the disease^[2]. Colorectal cancer is among the best characterized cancers with regard to the genetic progression of the disease^[3].

The ability to obtain maps of the protein content of cells provides a basis for comparison of the protein expression in normal and cancer cells. This capability becomes especially important for mapping the changes that occur during cancer progression. In the transformation process, changes in protein expression may result in expression of proteins at elevated or reduced levels^[4-6]. When a cell transforms from normal to malignant, changes in protein expression ultimately are reflected in the phenotype of the cell. It is also important to map the differences between different stages of cancer and between different cancer samples at the same stage of progression in order to understand the pathways involved in these processes. Ultimately, identification of important proteins involved in the transformation process may lead to the identification of early markers for detection of specific types of cancers and their treatments^[7].

In the present study, proteins from normal and cancerous colon tissues were extracted in three separate fractions. The mapping of a protein profile in each fraction was carried out individually. This approach eases the comparison of protein expression in normal and cancerous tissue as it reduces the total proteins present in each fraction. Using this method, we believe that proteins from different compartments of the cells were extracted in sequential order, where the aqueous soluble proteins were first extracted followed by non-aqueous soluble proteins consisting of proteins with moderate to extreme hydrophobicity. The proteins analyzed in this study were extracted from human tissue specimens (both cancerous and normal). In proteomics study, analysis of cancerous

Abstract

AIM: To identify and analyze the differentially expressed proteins in normal and cancerous tissues of four patients suffering from colon cancer.

METHODS: Colon tissues (normal and cancerous) were homogenized and the proteins were extracted using three protein extraction buffers. The extraction buffers were used in an orderly sequence of increasing extraction strength for proteins with hydrophobic properties. The protein extracts were separated using the SDS-PAGE method and the images were captured and analyzed using Quantity One software. The target protein bands were subjected to in-gel digestion with trypsin and finally analyzed using an ESI-ion trap mass spectrometer.

RESULTS: A total of 50 differentially expressed proteins in colonic cancerous and normal tissues were identified.

CONCLUSION: Many of the identified proteins have been reported to be involved in the progression of similar or other types of cancers. However, some of the identified proteins have not been reported before. In addition, a number of hypothetical proteins were also identified.

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Key words: Colon cancer; Tissue specimens; Sequential protein extraction; Proteomics

Gam LH, Leow CH, Man CN, Gooi BH, Singh M. Analysis of differentially expressed proteins in cancerous and normal

tissue has advantages over analysis in established cancer cell lines, as the latter may not represent the actual expression of proteins.

MATERIALS AND METHODS

Tissue specimen collection

Normal and cancerous colonic tissues were collected from patients with colon cancer. The specimens were provided by Hospital Pulau Pinang. Normal tissue was taken from the mucosal layer of the normal colon adjacent to the tumor tissue. Both the normal and cancerous tissues were confirmed by hospital pathologists. Ulceration or infected colon cancers were exclusion criteria for the study. The tissues were collected after informed consent had been obtained from the patients. The patients had different grades of colorectal cancer as shown in Table 1. The tissues were cut into similar sizes, weighed and immediately stored at -70°C until analyzed.

Well-differentiated adenocarcinoma refers to the less aggressive type of cancer where the cancerous tissues more closely resemble the normal tissue under histopathological examination, whereas moderately differentiated adenocarcinoma refers to moderately aggressive cancer cells.

Tissue lysis and sequential extractions

The tissues were defatted and an equal weight of tissues (normal and cancerous) was determined and subjected to analysis. The deep-frozen colon tissue specimens were disrupted by grinding in a liquid nitrogen-cooled mortar until the specimens became powder-form. The powder form-like tissues were divided into aliquots in separate eppendorf tubes. Sequential extraction of protein using three protein extraction buffers was carried out. These extraction buffers were prepared according to Molloy *et al*^[8] with modifications. The extraction procedures are described in the following sections.

Tris extraction buffer

The constituent of Extraction Buffer 1 (S1 buffer) is 40 mmol/L Tris. 1000 μL of S1 buffer was added to 500 mg of homogenized tissue. The mixture was vortexed for 30 seconds and centrifuged at 20°C with the speed of 12000 r/min for 8 min. The supernatant was collected in aliquots (50 μL) and kept at -70°C . The pellet was thoroughly washed with S1 buffer before being subjected to the second step in the sequential extraction.

Solution two extraction buffer

The extraction Buffer 2 (S2 buffer) contained 8 mol/L Urea, 50 mmol/L DTT, 40 g/L CHAPS, 2 mL/L Carrier ampholytes (pH 3-10) and 2 mg/L Bromophenol Blue. The recovered pellet was washed twice again with the T1 solution, and was then vortexed, centrifuged and the supernatant was discarded. After washing, a volume of 1000 μL S2 solution was added to the pellet. The mixture was then vortex for 30 s and then centrifuged at 20°C with the speed of 12000 r/min for 8 min. The supernatant was collected in aliquots (50 μL) and kept at -70°C . The pellet was thoroughly washed with the same extraction buffer before being subjected to the third step in the sequential

Table 1 Clinical and pathological data of patient tissue specimens

Patient #	Age	Gender	Race	Cancer stage	Cancer grade
MMN	77	Female	Malay	Stage IV	Well differentiated adenocarcinoma
LKH	66	Male	Chinese	Stage II	Moderately differentiated adenocarcinoma
CCH	55	Male	Chinese	Stage IV	Moderately differentiated adenocarcinoma
NSC	55	Female	Chinese	Stage III	Moderately differentiated adenocarcinoma

extraction.

Solution three extraction Buffer

The extraction Buffer 3 (S3 buffer) contained 5 M Urea, 2 mol/L Thiourea, 50 mmol/L DTT, 20g/L CHAPS, 20 mL/L Triton, 2 mL/L Carrier ampholytes (pH 3-10), 40 mmol/L Tris and 2 mg/L Bromophenol Blue. The recovered pellet from S2 extraction was washed twice with S2 buffer, it was then vortexed, centrifuged and the supernatant was discarded. After washing, 1000 μL of S3 buffer was added to the pellet. The mixture was vortexed for 30 s and then centrifuged at 20°C with the speed of 12000 r/min for 8 min. The supernatant was collected in aliquots (50 μL) and kept at -70°C . The pellet was then discarded.

Sample preparation and electrophoresis

The preparation of protein samples for SDS-PAGE separation was carried out by adding 200 mL/L of sample buffer (0.5 mol/L Tris-HCl, 100 mL/L glycerol, 20 mg/L SDS, 100 mg/L Coomassie blue) to each fraction (5 μL) and vortexed for 30 s. The samples (normal and cancerous) and the protein molecular weights markers were then loaded into individual wells of SDS-polyacrylamide gel using a syringe. Electrophoresis was performed using a vertical electrophoresis slab gel apparatus at a constant voltage of 200 volts throughout the electrophoresis process. Electrophoresis was terminated when the dye front was 2 to 3 mm away from the bottom edge of the gel for both experiments. The gel was stained with Coomassie blue.

In-gel digestion

The SDS-Polyacrylamide gel was washed thoroughly in 100 mmol/L NH_4HCO_3 and the differentially expressed protein bands from either normal or cancerous colonic tissues were then excised from the gel. In-gel digestion was performed using trypsin according to Gam and Aishah^[9] with slight modification. The gel pieces were first excised and shrunk by dehydration in acetonitrile. The solvent was then discarded and the gel pieces were dried in a vacuum centrifuge. A volume of 10 mmol/L dithiotreitol (DTT) in 100 mmol/L NH_4HCO_3 that was sufficient to cover the gel pieces was added and the protein was reduced

for 1 h at 56°C. After cooling to room temperature, the DTT solution was replaced with the same volume of 55 mmol/L iodoacetic acid in 100 mmol/L NH₄HCO₃. After 45 min incubation at ambient temperature in the dark with occasional vortexing, the gel pieces were washed with 50-100 µL of 100 mmol/L NH₄HCO₃ for 10 min, dehydrated with acetonitrile, rehydrated in 100 mM NH₄HCO₃ and dehydrated in the same volume of acetonitrile. The liquid phase was removed and the gel pieces were dried in a vacuum centrifuge. The gel pieces were swollen in digestion buffer containing 50 mmol/L NH₄HCO₃, 5 mmol/L CaCl₂, and 12.5 ng/µL of trypsin in an ice-cold bath. After 45 min, the supernatant was removed and replaced with 10 µL of the same buffer but without trypsin to keep the gel pieces wet during enzymatic cleavage at 37°C overnight. Peptides were extracted from the gel matrix by adding 15 µL of 20 mmol/L NH₄HCO₃, vortexed and incubated at room temperature for 10 min and the supernatant was recovered after a brief spin. This was followed by adding (1 to 2 times the volume of gel pieces) 50 mL/L formic acid in acetonitrile:water mixture (70:30), vortex and incubated for 20 min at room temperature. It was then spun down and the supernatant was recovered. These steps were repeated 3 times. Pooled extracts were dried in a vacuum centrifuge and stored at -70°C. Each of the pooled extracts (peptides) was added with 30 µL of deionized water. Then, they were centrifuged at 500 r/min for 5 min at 15°C. The extracts were then subjected to LC/MS/MS analysis.

Mass spectrometric analysis

A volume of 5 µL of the sample was injected into a RPC-column (C₁₈ 300 A, 5 µm, 1 mm × 150 mm) connected to a Hewlett Packard HPLC. A capillary pump was used to pump the mobile phase at 20 µL/min flow rate, the linear gradient used was 5% B to 95% B in 65 min. Mobile phase A was 500 µL/L formic acid in deionized water and B was 500 µL/L formic acid in ACN. The HPLC was interfaced to an ion trap mass spectrometer. The dry gas temperature was 300°C, dry gas flow rate was of 8.0 L/min, nebulizer pressure of 30.0 psi. The above parameters were used subsequently for acquiring MS data. The peptides were ionized using electrospray soft ionization technique (ESI).

The experimental method was made up of one experimental protocol consisting of 2 scan events. The first scan event was a full scan MS and the second was the data dependent MS/MS scan which is dependent on the results of the first scan event. The most intense ion in a MS scan will be automatically isolated and excited to MS/MS scan. The parameters set for data dependent scan (MS/MS scan) were default collision energy (voltage) = 1.15 V, charge state = 2, minimum threshold = 3000 counts, and the isolation width = 2 m/z.

Mascot protein identification

The protein was identified using Mascot Protein Database Search engine. The Peptide Mass Tolerance was set as ± 2 u and ± 0.8 u was set for the Fragment Mass Tolerance. Only 1 missed cleavage was allowed. This software is available at www.matrix-science.com. Matches were computed using a probability-based Mowse score defined as $-10 \times$

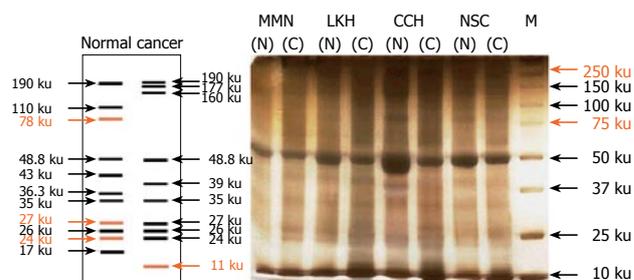


Figure 1 Proteins profiles of normal and cancer colon tissues from four different patients. The proteins were extracted using in S1 buffer. N: Normal colon tissues; C: Cancer colon tissues; M: Marker; Lanes 1 and 2: MMN samples; Lanes 3 and 4: LKH samples; Lanes 5 and 6: CCH samples and Lanes 7 and 8: NSC samples. Diagram 1 shows the consistent protein bands found in the normal and the cancer tissues of the figure.

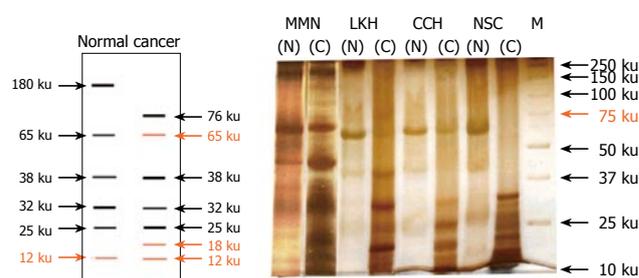


Figure 2 Proteins profiles of normal and cancer colon tissues from four different patients. The proteins were extracted using in S2 buffer. N: Normal colon tissues; C: Cancer colon tissues; M: Marker; Lanes 1 and 2: MMN samples; Lanes 3 and 4: LKH samples; Lanes 5 and 6: CCH samples and Lanes 7 and 8: NSC samples. Diagram 2 shows the consistent protein bands found in the normal and the cancer tissues of the figure.

$\log(P)$, where P is the probability that the observed match was a random event.

RESULTS

Figure 1 shows the protein profiles of protein extracted using S1 buffer from normal and cancerous colon tissues of four different patients. Diagram 1 shows the consistent protein bands for both normal and cancerous tissues. The thick lines represent the protein bands that were expressed in all four tissues and the faint lines represent the protein bands that were expressed in three of the four patients. The distinct bands that were only detected in one particular tissue are ignored, as it may not represent the proteins that are involved in cancer formation. In colon cancer tissues, the protein bands that were expressed in all the four patients were at 190 ku, 177 ku, 160 ku, 48.8 ku, 39 ku, 35 ku, 27 ku, 26 ku and 24 ku. However, a protein band at 11 ku was detected in three of the four patients. In normal colon tissues, protein bands at 190 ku, 110 ku, 48.8 ku, 43 ku, 36.3 ku, 35 ku, 26 ku and 17 ku were found in all the patients' tissues while protein bands at 78 ku, 27 ku and 24 ku were found expressed only in three patients. When comparing cancerous and normal tissue, it is clear that some of the proteins were differently expressed between the two types of tissues.

Figure 2 shows the profiles of proteins extracted using S2 buffer from both normal and cancerous colonic tissues.

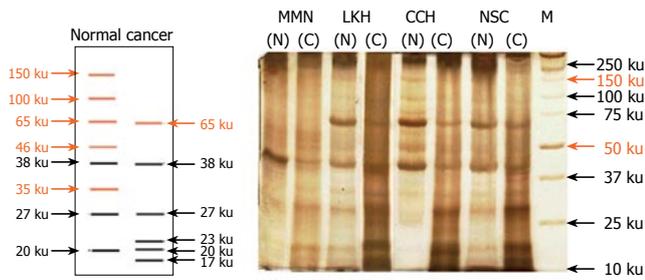


Figure 3 Proteins profiles of normal and cancer colon tissues from four different patients. The proteins were extracted using in S3 buffer. N: Normal colon tissues; C: Cancer colon tissues; M: Marker; Lanes 1 and 2: MMN samples; Lanes 3 and 4: LKH samples; Lanes 5 and 6: CCH samples and Lanes 7 and 8: NSC samples. Diagram 3 shows the consistent protein bands found in the normal and the cancer tissues of the figure.

Table 2 Up-regulated, down-regulated proteins extracted in S1, S2, S3 buffers from colon tissues

SWISS-PROT accession number	Down-regulated proteins (Normal tissues)	SWISS-PROT accession number	Up-regulated proteins (Cancer tissues)
P12718	Actin, gamma-enteric smooth muscle	P04262	Type II, keratin subunit protein
P17661	Desmin	P35900	Keratin 20
P08670	Vimentin	P35908	Keratin, 67k type II epidermal
Q9DFD0	Hypothetical protein	P13647	Keratin k5
Q7Z5W1	Hypothetical protein	Q86Y46	Keratin 6 irs 3
CAD19027	Hypothetical protein	P04264*	Keratin 1, type II cytoskeletal
BAC87457	Hypothetical protein	P35527	Cytoskeletal 9
CAC08866	Hypothetical protein	P04264	Keratin, type II cytoskeletal I
CAC08835	Hypothetical protein	P35527	Keratin 9, type I, cytoskeletal
Q8WTU6	Hypothetical protein	P08779	Keratin, type 1, cytoskeletal 16
Q01995	Transgelin	P48669	Keratin, type II, cytoskeletal 6F
Q91706	Skeletal muscle beta-tropomyosin	P02538	Keratin 6A, type II
P12277	Creatine kinase	P19013	Keratin, type II, cytoskeletal 4
P00915	Actin-gamma	P08729	Keratin, type II, cytoskeletal 7
Q96QE3	ATP[GTP]-binding protein	P12035	Keratin, type II, cytoskeletal 3
		P05783	Keratin, type I, cytoskeletal 18
		P13645	Keratin, type I, cytoskeleton 10
		Q8NF17	Hypothetical protein
		CAE99935	Hypothetical protein
		P02675	Fibrinogen beta chain
		P00915	Carbonate dehydratase EC 4.2.1.1
		Q9PTK9	Kinesin-like protein 2
		P60174	Triosephosphate isomerase EC. 5.3.1.1
		Q6PJ43	ACTGI protein
		Q13733	Sodium/potassium-transporting ATPase alpha-4 chain EC 3.6.3.9

Diagram 2 represents the consistent protein bands that were expressed in the tissues analyzed. For the cancerous tissues, the protein bands at 76 ku, 38 ku, 32 ku and 25 ku were consistently expressed in all the cancerous tissues analyzed while protein bands at 65 ku, 18 ku and 12 ku were only found in the tissues of three of the four patients. For the normal tissues, the bands at 180 ku, 65 ku, 38 ku, 32 ku and 25 ku were found in the tissues of all four patients while only one band at 12 ku was detected in the tissues of three patients. Most of the proteins extracted from cancerous tissues using S2 buffer were found at the low molecular weight region, in the range of 35 ku to 10 ku. In contrast, relatively few protein bands from normal tissues were detected in this region and those that were detected were present at low protein intensity.

Figure 3 shows the protein profiles of proteins extracted using S3 buffer from normal and cancer colon tissues of the four patients. Diagram 3 represents the

consistent protein bands expressed in the four tissues analyzed. Protein bands at 38 ku, 27 ku, 23 ku 20 ku and 17 ku from cancerous tissues were found in all four patients whereas only the band at 65 ku was found in three of the four patients. Normal tissues of the corresponding patients displayed bands at 38 ku, 27 ku and 20 ku whereas tissues from three patients were found to express consistent bands at 150 ku, 100 ku, 65 ku, 46 ku and 35 ku. The protein band at 65 ku was detected at the highest intensity compared to the other bands and it was uniquely expressed in all the patients except for patient #MMN, which was the only patient diagnosed with well differentiated adenocarcinoma cancer. Thus, its expression is probably related to the cancer grade. Nevertheless, this may just be an ambiguous assumption as only one patient with well differentiated cancer grade was analyzed. When comparing the protein profiles of protein extracted from tissues using S3 buffer, it is obvious that the expression of

Table 3 Unique proteins extracted in S1, S2, S3 buffers from colon tissues

SWISS-PROT accession number	Unique proteins (Normal tissues)	SWISS-PROT accession number	Unique proteins (Cancer tissues)
P32119	Peroxiredoxin 2 precursor	Q9BX84	LTRPC6 channel kinase 6
Q03001	Bullous pemphigoid antigen 1 precursor	Q9AVW8	DNA-directed RNA polymerase II largest chain EC 2.7.7.6
P01009	Alpha-1-antitrypsin precursor	P29312	14-3-3 protein zeta
P20848	Alpha-1-antitrypsin related protein precursor	P31946	14-3-3 protein beta/alpha
1QMBA	Alpha-1-antitrypsin mutant YES, chain A	S38532	Protein 14-3-3 eta chain
		S31975	14-3-3 protein epsilon

low molecular weight proteins (below 37 ku) in cancerous tissues was greater than that of the normal tissues.

Table 2 shows the list of proteins detected, with a total of 15 and 24 down-regulated and up-regulated proteins identified from normal and cancerous tissues, respectively. Table 3 shows the unique proteins that were identified from normal and cancerous tissues.

DISCUSSION

The sequential protein extraction technique consists of three types of protein extraction buffers, namely the S1 buffer, S2 buffer and S3 buffer. The S1 buffer is used mainly to extract aqueous soluble proteins, such as cytosolic proteins and nuclear proteins while the S2 and S3 buffers were used to extract proteins with intermediate to extreme hydrophobicity. The constituent of S2, which is made up of chaotropic agents and other reagents may disturb rugged protein-protein interaction found among structural, cytoskeletal proteins and aggregating proteins^[10]. However, the combination of urea and thioureas in S3 was used to enhance the solubility of membrane proteins^[8]. The extraction buffers were always used in the orderly sequence of S1 followed by S2 and then S3. The use of sequential protein extraction buffers was first suggested by Molloy *et al.*^[8] for separation of protein mixtures using 2D-gel electrophoresis. However, in our present study, the extracted proteins were separated using the SDS-PAGE technique, whereby direct comparison of protein profiles from tissues of four different patients was carried out.

In general, use of sequential extraction buffers is beneficial to this study because proteins were extracted according to their increasing hydrophobic strength, which may imply that proteins from different cellular compartments were extracted in different fractions. This method not only allows the analysis of proteins according to their localization in the cells but also reduces the number of proteins extracted in each fraction, which provides better resolution of proteins and their visualization in SDS-PAGE.

The protein profiles of the proteins extracted using the S1 and S2 buffers are significantly different, however the protein profiles of proteins extracted using S2 and S3 showed only slight differences. The significant variation between the protein profiles of S1 and S2 can be explained by the vast difference in the property of the proteins extracted, where S1 buffer extracts proteins only solubilize in aqueous solution while S2 buffer extracts the non-

aqueous soluble proteins. The protein profiles displayed by proteins extracted using S2 and S3 also showed a certain degree of variation as S3 buffer was used to enhance the solubility of more stringent proteins from the tissue^[11].

The differentially expressed proteins in each of the gels were then subjected to further analysis and the identity of each protein was analyzed using LC/MS/MS. Proteins were identified using the Mascot protein search engine on the basis of peptide mass matching^[12] with redundancy of post-translational modification and proteolysis. The definition of “up-regulated”, “down-regulated” and unique proteins in this study are solely dependent on their existence in SDS-PAGE. When analyzed using Quantity one software (BioRad), the intensity of the bands for up-regulated proteins was more intense in cancerous as compared to normal tissues while the “down-regulated” proteins were the proteins that were more intense in normal as compared to cancerous tissues. However, some of the intense protein bands in SDS-PAGE were found to contain more than one type of protein. Therefore, for the up-regulated and down-regulated protein bands, both the protein bands of the identical molecular weight from normal and cancerous tissues were excised and subjected to LC/MS/MS analysis. The proteins that were detected in both tissues were then quantified by their peptides’ peak areas in selected ion chromatogram analysis. The proteins present in one tissue and not the other are reported as unique proteins.

A total of ninety-five proteins were identified from the protein bands indicated earlier for both the normal and cancerous tissues. However, some of these proteins were of serum origin, due to the embedded blood vessels in the tissues, and these proteins were excluded from the protein list. Furthermore, some of the proteins were represented by more than one band as described by heterogeneity. The heterogeneous proteins were mainly derived from structural, transport and enzyme subunits such as Actin, gamma-enteric smooth muscle, Desmin, Vimentin, Keratin, type I cytoskeletal 10, Cytoskeletal 9, and Triosephosphate isomerase EC 5.3.1.1. Phosphorylation, glycosylation and limited proteolytic activities have been considered as major modification of proteins and they therefore affect the mobility of the modified proteins in SDS-PAGE^[13-15].

The structural proteins were mostly extracted from the cancerous tissues using the S3 buffer. The majority of the structural proteins identified belongs to the cytokeratin family. Williams *et al.*^[16] reported that cytokeratin expressing tumor cells contain cytokeratins 8 and 18, but not

cytokeratin 19 or vimentin. The roles of cytokeratins are commonly known to encompass both structural and signaling capabilities. In the present study, cytokeratin 18 was found in cancerous colon tissues in S3 buffer extracts. The expression of cytokeratin 18 in colonic cancerous tissues is consistent with those reported by Leong *et al*^[17]. In contrast, the expression of cytokeratin 18 was found reduced in malignant tumors of prostate carcinoma compared to benign prostatic hyperplasia^[4]. The expression of cytokeratins is sometimes used as an indicator of the behavioral changes of a tissue. For instance, in breast tissue, cytokeratin 8 has been reported to be the major receptor for plasminogen on breast cells^[18].

Vimentin is expressed at higher levels in the low-secreting variant. It is present in many cell lines, but normally absent from differentiated cells. Thus, lower vimentin expression in the high secretors may be indicative of a more differentiated phenotype^[19]. In our present study, vimentin is a down-regulated protein found in S1 buffer extracts. Williams *et al*^[16] has reported that the absence of vimentin in colon cancer tissue is due to its correlation with cytokeratin 19 or other cytokeratin expressions. However, Birchmeier *et al*^[20] and Seshadri *et al*^[21] reported no unequivocal correlation between a gain of vimentin and poor prognosis with primary tumors.

Hypothetical proteins were extracted in all three extraction buffers from both normal and cancerous colonic tissues. Generally, hypothetical proteins are defined as the proteins that were not described at the protein level, but were predicted from cDNA sequences^[22]. As shown in Table 2, a series of hypothetical proteins were identified. Afjelhi-Sadat *et al*^[23] suggested that the hypothetical proteins may serve as marker or protein vaccine candidates. Particularly when the hypothetical protein is found in cancerous tissue exclusively, it can be considered as novel and of pivotal importance. Some of the hypothetical proteins were detected as heterogeneous proteins suggesting the post-translational nature of the proteins.

Peroxiredoxin 2 is a type transport protein and it was extracted in the S1 buffer as a unique protein in normal tissues. Peroxiredoxin 2 is part of the peroxidase family, which are proteins that have been known to connect with cell proliferation, differentiation and apoptosis.

Fibrinogen is a type of channel protein and its role was reported to be supporting the binding of growth factors and promoting the cellular responses of adhesion, proliferation, migration during angiogenesis and tumor cell growth^[24]. Fibrinogen was detected in the S2 buffer of cancerous tissues. The presence of fibrinogen in cancer cells was demonstrated to affect the progression of tumor cell growth and metastasis on the basis that fibrinogen alters the ability of breast cancer cells to migrate^[24]. In addition, fibrinogen was also found present in lung cancer following the detection of elevated plasma fibrinogen levels in advanced stages of the disease^[25] and the localization of fibrinogen in tumor tissue^[26,27]. There are also reports of the deposition of fibrinogen on the surfaces of tumor cells and lymphocytes^[28,29]. In primary and metastatic tumor^[30], the presence of fibrinogen was regarded as an adverse event leading to tumor

development. Yamaguchi *et al*^[31] reported that lung cancer was developed through induction of hyperfibrinogenemia.

Transgelin (SM22-alpha) is a cytoskeletal-binding protein that was isolated in S1 buffer as a down-regulated protein. Maguire *et al*^[32] has reported that loss of transgelin expression is an indication of early tumor progression and the authors suggested that transgelin may serve as a diagnostic marker for breast and colon cancer. We found that the expression of transgelin in cancerous tissue is much lower than that of normal tissue. Other cytoskeletal-binding proteins that were detected in S1 buffer extract were Bullous pemphigoid antigen 1 precursor and Skeletal muscle beta-tropomyosin.

Some of the enzymes that play important roles in metabolic pathways and are essential for energy production were detected as up-regulated protein in S1 buffer. These enzymes include triosephosphate isomerase and LTRPC6 channel kinase 6. LTRPC6 channel kinase 6 is an essential ion channel and serine/threonine-protein kinase is crucial for magnesium homeostasis. It plays an important role in epithelial magnesium transport and in the active magnesium absorption in the gut and kidney.

Sodium/potassium-transporting ATPase alpha-4 chain was detected as the up-regulated protein in S3 buffer. It is an integral membrane protein that is located in the cell membrane^[33]. It catalyzes the hydrolysis of ATP coupled with the exchange of sodium and potassium ions across the plasma membrane.

Carbonate dehydratase is also the up-regulated protein detected in S1 and S2 buffer, it is a zinc-containing enzyme that catalyses the reversible hydration of carbon dioxide, which is significant in the transport of CO₂ from the tissues to the lungs. It was reported that over expression of the zinc enzyme carbonic dehydratase is observed in certain human cancers^[34]. Its expression was reported to be elevated in the renal cancer cells as compared to the surrounding normal kidney tissue^[35].

DNA-directed RNA polymerase II largest chain is the DNA-RNA binding protein that was detected as unique protein in S1 buffer extract of cancer tissues. It was also reported in human brain tumors^[36].

Proteins 14-3-3 are the protein-binding proteins that were isolated in the S1 buffer as unique proteins from colon cancer tissue. Protein 14-3-3 plays a role in the regulation of signal transduction protein pathways implicated in the control of cell proliferation, differentiation and survival^[37]. This protein was reported to play multiple roles in maintaining cell survival^[38,39]. Friedman *et al*^[40] has reported that protein 14-3-3 in three different isoforms was expressed up to 1.7 fold in the colon tumors as compared to normal colon tissue.

Alpha-1-antitrypsin is an endopeptidase inhibitor protein that was isolated as a unique protein in S1 buffer from normal tissue only. In individuals who lack this inhibitor protein, the protease destroys the membrane system, leaving the colon and rectum vulnerable to colorectal cancer development^[41]. The main role of alpha-1-antitrypsin is in defense against elastase damage that occurs in the lung under normal physiology conditions^[42]. Alpha-1-antitrypsin was detected in patients with lung cancer^[43] and was used as a cancer marker for cervical

cancer^[44]. Contrary to our finding, Friedman *et al.*^[40] reported the detection of alpha-1-antitrypsin in colon cancer.

ATP (GTP)-binding protein is ATP-binding protein that was detected as down-regulated protein in S2 buffer. It is a mitochondrial membrane protein.

Kinesin is a motor activity protein that was detected as an up-regulated protein in S2 buffer extract. It is a microtubule-associated force-producing protein that may play a role in organelle transport^[45]. This protein was also reported in breast cancer tissue^[46]. Kinesin-like protein 2 is an immunogenic breast cancer antigen^[47]. In addition, kinesin-like protein 2 was also reported in pancreatic cancer^[48].

ACTG1 is another type of motor activity protein that was detected as an up-regulated protein. It is a protein involved in the formation of filaments, which is the major component of the cytoskeleton^[49]. Its existence in colorectal cancer was also reported by Vadlamudi and Shin^[50]. Alteration of cytoskeletal proteins may have an important role in cancer initiation or progression. Mutations affecting four major cytoskeletal components have now been identified in human neoplastic cells. Chou *et al.*^[51] postulated that mutated cytoskeletal genes may be members of a class of oncogenes, which are fundamentally different from both the nuclear-acting and growth factor/receptor/protein kinase-related types of oncogenes.

In this study, a number of the differentially expressed proteins extracted from the normal and cancerous colonic tissues were identified. Amongst the proteins identified, some of the proteins have their roles identified in similar or other types of cancers. There are also a series of hypothetical proteins with unknown functions. Some of these proteins have their primary location at the cell membrane, which may serve as potential antigens for drug-targeted therapy or as candidates for vaccines against colon cancer. These proteins are under further investigation in our laboratory.

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Inhibitory effect of caffeic acid phenethyl ester on the growth of SW480 colorectal tumor cells involves β -catenin associated signaling pathway down-regulation

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gene expression may mediate the anti-tumor effects of CAPE.

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Abstract

AIM: To study the anti-tumor effect of caffeic acid phenethyl ester (CAPE) and the influence of CAPE on β -catenin associated signaling pathway in SW480 colorectal cancer (CRC) cells.

METHODS: SW480 cells were treated with CAPE at serial concentrations. The proliferative status of cells was measured by methabenzthiazuron (MTT) assay. Cell cycle and cell apoptosis were analyzed using flow cytometry (FCM). Western blotting assay was used to evaluate the protein level of β -catenin, c-myc and cyclinD1. β -catenin localization was determined by indirect immunofluorescence.

RESULTS: CAPE displayed a strong inhibitory effect in a significant dose- and time-dependent manner on SW480 cell growth. FCM analysis showed that the ratio of G0/G1 phase cells increased, S phase ratio decreased and apoptosis rate increased after SW480 cells were exposed to CAPE for 24 h. Pretreatment of SW480 cells with CAPE significantly suppressed β -catenin, c-myc and cyclinD1 protein expression. CAPE treatment was associated with decreased accumulation of β -catenin protein in nucleus and cytoplasm, and concurrently increased its accumulation on the surface of cell membrane.

CONCLUSION: CAPE can inhibit SW480 cell proliferation by inducing cell cycle arrest and apoptosis. Decreased β -catenin and the associated signaling pathway target

INTRODUCTION

Caffeic acid phenethyl ester (CAPE) is a phenolic anti-oxidant. As an active component of propolis, CAPE has many biological and pharmacological functions including immunoregulation, anti-inflammatory, antiviral, antibacterial and antitumor activities. Several studies have demonstrated that CAPE has antiproliferative effect, apoptosis-inducing effect in various tumor cells *in vitro*^[1-4] and *in vivo*^[5,6]. CAPE also inhibited the development of azoxymethane-induced aberrant crypts in the colon of rats^[7]. Furthermore, a number of studies reported that CAPE elicits apoptosis and suppresses the growth of transformed cells, and the cytotoxicity of CAPE to transformed cells is sensitive and selective^[8-10]. Multiple molecular mechanisms seem to be involved in the tumor suppressive effects of CAPE. It was reported that CAPE could inhibit NF κ B and induce apoptosis *via* Fas signal activation in human breast cancer MCF-7 cells^[11]. Additionally, Carrasco *et al*^[12] found an 85% decrease in nuclear localization of the p65 subunit of NF-kappa B. While in C6 glioma cells, the tumor suppressor proteins P53 and p38 mitogen-activated protein kinase (p38 MAPK) are involved in CAPE-induced apoptotic cell death^[13].

Colorectal cancer (CRC) is the third most common cancer and the fourth most frequent cause of cancer death worldwide. Recent studies have indicated that in most of CRC, there are aberrant β -catenin associated signaling pathways^[14]. The β -catenin associated signaling pathway

plays critical roles in cell proliferation and differentiation, and the β -catenin is the central component in the pathway. Aberrant β -catenin associated signaling pathway, which generally results from inactivating mutation of adenomatous polyposis coli (APC) or activating mutation of β -catenin, leads to the accumulation of β -catenin in the nucleus of cells, which is subsequently complexed with T-cell factor (TCF) and promotes transcription of a variety of target genes, such as c-myc, cyclinD1, ultimately leading to cell aberrant proliferation and tumor formation. Dysregulation of β -catenin associated signaling and hence β -catenin expression is believed to be central to the early stages of sporadic colorectal carcinogenesis in humans^[15-17]. Therefore, control of β -catenin and/or control of the downstream target gene expression represents an opportunity for rational colorectal cancer therapy.

Here we report the anti-tumor effect of CAPE in association with β -catenin associated signaling in SW480 colorectal cancer cells. These studies have important implications for CAPE to be used as a potential therapeutic agent for colorectal cancer.

MATERIALS AND METHODS

Chemicals

CAPE, dimethyl sulphoxide (DMSO), PI, methanbenzthiazuron (MTT), mouse anti-human β -catenin, c-myc, cyclinD1 and β -actin monoclonal antibody were purchased from Sigma-Aldrich (USA). RPMI-1640 medium, fetal calf serum and trypsin-EDTA were purchased from Hyclone (USA). FITC and horseradish peroxidase-conjugated secondary antibody were obtained from Pierce Biotechnology (USA). Annexin-V and PI double staining kit and Tripure were purchased from Roche (Germany). Protein assay kits were obtained from Bio-Rad Labs (USA). Enhanced chemiluminescence (ECL) system was purchased from Amersham Life Science (UK). CAPE was dissolved with DMSO and adjusted to final concentrations with culture medium prior to use.

Cell culture

The human CRC cell line SW480 was purchased from the American Type Culture Collection (ATCC). The cells were cultured in RPMI-1640 medium supplemented with penicillin G (100 U/mL), streptomycin (100 μ g/mL) and 10% fetal calf serum. Cells were grown at 37°C in a humidified atmosphere of 50 mL/L CO₂ and were routinely subcultured using 0.25% (w/v) trypsin-EDTA solution.

MTT assay

The logarithmically growing SW480 cells were plated into a 96-well plate at a density of 4×10^3 cells/well. After 24 h, the cells were treated with CAPE at designated concentrations (2.5, 5, 10, 20, 40 and 80 mg/L) for 24, 48, 72 and 96 h, respectively. Control wells were treated with 0.1% DMSO alone. Then, 20 μ L MTT (5 g/L) was added to each well and incubated for an additional 4 h, and then culture media were discarded followed by addition of 0.15 mL DMSO and vibration for 10 min. The absorbance was measured at 490 nm. The inhibitory rates (IR) were calcu-

lated as follows: IR (%) = [(1-absorbance of the treated wells)/(absorbance of the control wells)] \times 100%. The 50% inhibitory concentration (IC₅₀) value was determined using CalcuSyn software.

Flow cytometry analysis

Cell density was adjusted to $0.3-1.0 \times 10^7$ cells/mL. Cells were serum-starved for 24 h and then treated with different concentrations of CAPE (2.5, 5 and 10 mg/L) for 24 h. Then the cells were harvested with trypsin-EDTA to produce a single cell suspension. The cells were pelleted by centrifugation and washed twice with phosphate-buffered saline (PBS). Then, the cell pellets were resuspended in 0.5 mL PBS and fixed in 5 mL ice-cold 70% ethanol at 4°C. The fixed cells were spun down by centrifugation and the pellets were washed with PBS. After resuspension with 1 mL PBS, the cells were incubated with RNase A (20 mg/L) and PI (50 mg/L) and shaken for 1 h at 37°C in the dark. The stained cells were analyzed using a FACScan flow cytometer in combination with BD analysis II software (Becton Dickinson).

Annexin-V and PI double-staining flow cytometry analysis

The cells were treated and harvested in the same way as mentioned above. Analysis of apoptosis was performed using the Annexin-V and PI double staining kit according to the manufacturer's protocol. After treatment with CAPE, the cells were immediately analyzed by flow cytometry. The apoptotic cells were localized in the right lower quadrant of a dot-plot graph by using Annexin-V-fluorescein versus PI.

Western blot analysis

Cells were placed in serum-free medium with or without CAPE (2.5, 5 and 10 mg/L) for 24 and 48 h, then were lysed in solubilization buffer containing sodium dodecyl sulfate (SDS) and β -mercaptoethanol. After centrifugation, the supernatant was collected and the protein concentration determined by the BioRad DC kit. Equal amounts of protein (50 μ g) in each sample were resolved in 10% SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane. After blocked with 2% skim milk, the membranes were incubated with primary antibodies at appropriate dilution (β -actin, 1/1000; β -catenin, c-myc and cyclinD1, 1/500) overnight at 4°C. Horseradish peroxidase-conjugated secondary antibody was diluted 1/5000 and incubated for 1 h at 20°C. Immunoreactive proteins were detected with an ECL system, and light emission was captured on Kodak X-ray films. Product bands were quantitated by scanning densitometry.

Indirect immunofluorescence

SW480 cells grown on glass coverslips were treated for 48 h with CAPE (2.5, 5 and 10 mg/L) or an equivalent dilution of DMSO, under standard culture conditions as described above. Then cells were washed with PBS and fixed with methanol for 20 min. Incubation with monoclonal anti- β -catenin antibody (1:200) was carried out overnight at 4°C. This was followed by incubation with FITC-

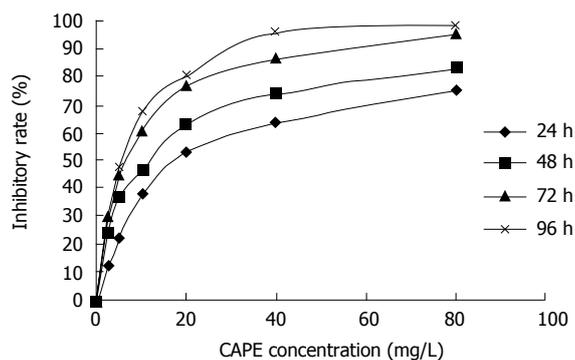


Figure 1 Effect of CAPE on SW480 cell proliferation. SW480 cells were treated with CAPE at indicated concentrations for 24, 48, 72 and 96 h. The cell viability was determined by MTT assay.

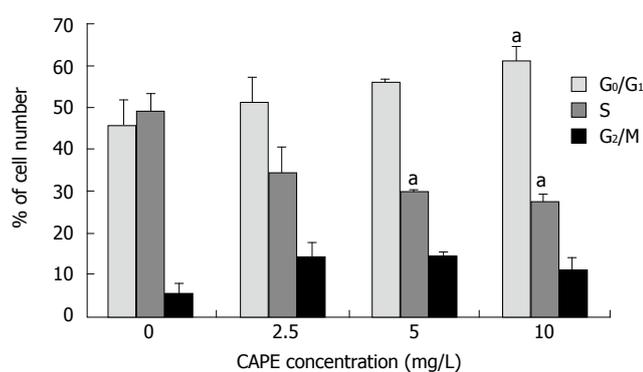


Figure 2 Effect of CAPE on SW480 cell cycle distribution. The percentage of cells in each phase of the cell cycle, G₀/G₁, S, and G₂/M was determined by FCM after cells were treated with indicated concentrations (2.5, 5 and 10 mg/L) of CAPE for 24 h. ^a*P* < 0.05 vs the control group, treated with vehicle, DMSO only.

conjugated secondary antibody (1:200) for 1 h at room temperature. Omission of the primary antibody was used as a negative control. Images were collected using laser scanning confocal microscopy.

Statistical analysis

Data were expressed as mean \pm SD. Analysis of data was performed using one-way ANOVA. *P* < 0.05 was considered statistically significant.

RESULTS

Effect of CAPE on cell proliferation

SW480 cells were treated with various concentrations (2.5, 5, 10, 20, 40 and 80 mg/L) of CAPE for 24, 48, 72 and 96 h, respectively. Then the cell viability was measured by MTT assay. CAPE showed a significant dose-dependent and time-dependent inhibition of cell growth (Figure 1). The IC₅₀ value for CAPE at 24, 48, 72 and 96 h after treatment was 20.27, 11.38, 6.15 and 5.44 mg/L, respectively.

Effects of CAPE on cell cycle progression

In order to examine the effects of CAPE on cell cycle progression, SW480 cells were treated with designated concentrations of CAPE for 24 h. The distributions of cells in each of the cell cycle phases were determined by FCM. As shown in Figures 2 and 3, the percentage of cell

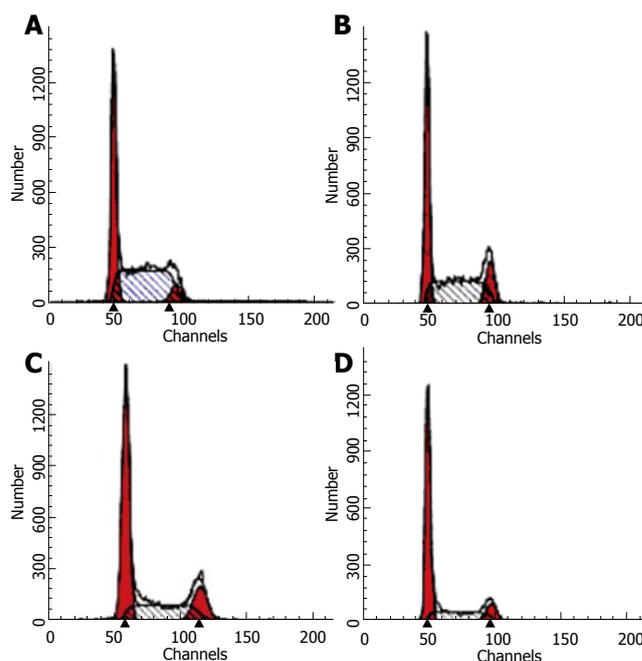


Figure 3 Flow cytometry analysis of SW480 cells following 24 h incubation with CAPE (A) 0 mg/L, (B) 2.5 mg/L, (C) 5.0 mg/L, (D) 10 mg/L.

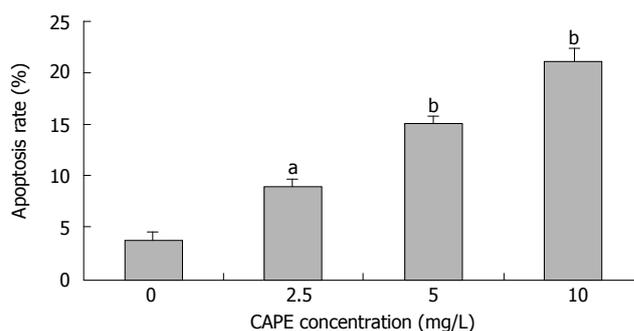


Figure 4 Effect of CAPE on SW480 cell apoptosis. After treated with CAPE at indicated concentrations for 24 h, the SW480 cell apoptosis rates were analyzed by Annexin-V and PI double-staining flow cytometry. ^a*P* < 0.05, ^b*P* < 0.01 vs the control group.

population of the G₀/G₁ phase significantly increased, while those in S and G₂/M phase decreased. CAPE caused cell cycle arrest in G₀/G₁ phase in a dose-dependent manner.

Effect of CAPE on apoptosis

After SW480 cells were exposed to CAPE at various concentrations (2.5, 5 and 10 mg/L) for 24 h, Annexin-V and PI double-staining flow cytometry analysis showed that the apoptosis rates were 9.1% \pm 0.7%, 15.23% \pm 0.6% and 21.1% \pm 1.44%, respectively, which were significantly higher than that in the control group (3.7% \pm 0.9%). CAPE induced apoptosis of SW480 cells in a dose-dependent manner (Figures 4 and 5).

Effect of CAPE on the expression of β -catenin, c-myc and cyclinD1

To investigate the mechanisms underlying CAPE induced apoptosis, we examined the effect of CAPE on the expres-

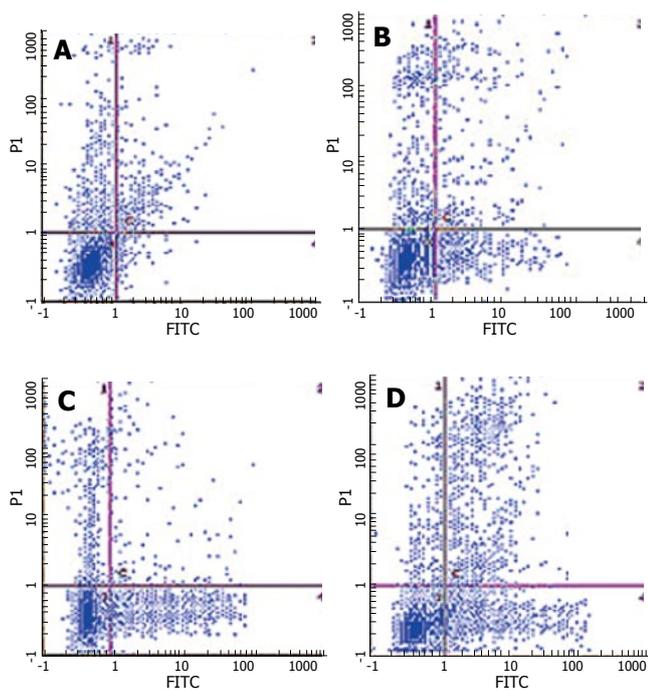


Figure 5 Annexin-V and PI double-staining flow cytometry analysis of SW480 cells following 24 h incubation with CAPE (A) 0 mg/L, (B) 2.5 mg/L, (C) 5.0 mg/L, (D) 10 mg/L.

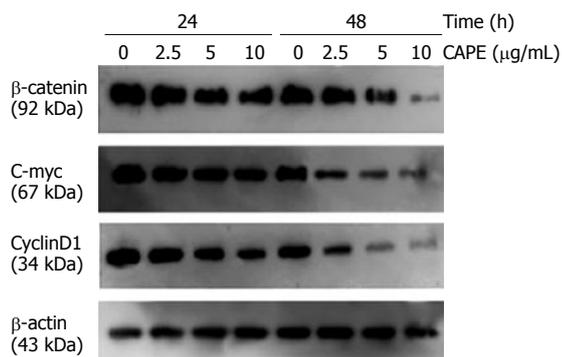


Figure 6 Western blot analysis of β -catenin, c-myc and cyclinD1 protein expression in SW480 cells following 24 and 48 h incubation with indicated concentrations of CAPE. β -actin served as a loading control.

sion of β -catenin signaling pathway related genes. After SW480 cells were exposed to CAPE (2.5, 5 and 10 mg/L) for 24 and 48 h, Western blot analysis showed that CAPE significantly suppressed β -catenin protein expression in a dose and time-dependent manner. CAPE treatment after 48 h markedly decreased the expression of c-myc and cyclin D1, two of the β -catenin associated signaling pathway target genes (Figure 6).

Indirect immunofluorescence studies of β -catenin localization in SW480 cells revealed that CAPE treatment decreased the accumulation of β -catenin protein in nucleus and cytoplasm, and concurrently increased its accumulation on the surface of cell membrane (Figure 7).

DISCUSSION

Propolis has been used since ancient times in folk

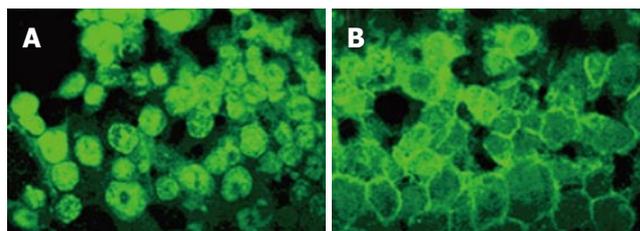


Figure 7 Indirect immunofluorescence analysis of β -catenin protein localization in SW480 cells following 48 h incubation with CAPE (A) 0 mg/L, (B) 10 mg/L.

medicine. Researches indicate that propolis exhibits immunoregulation, anti-bacterial, anti-inflammatory and anti-tumor activities^[18-20]. As an active component of propolis, CAPE is receiving much attention in the medical research community because of its potential for the treatment of a number of disorders, including cancer. Recent studies demonstrated that CAPE has antiproliferative and apoptosis-inducing effects in various tumor cells. An *in vivo* study showed that CAPE decreased the growth of the xenografts of C6 glioma cells in nude mice^[6]. Also, dietary intake of CAPE decreased tumor formation in the enterocytes of the Min/+ mouse^[21].

In the present study, we investigated the effect of CAPE on cell proliferation, cell cycle and apoptosis of SW480 CRC cells. Our data demonstrated that CAPE treatment could significantly inhibit cell growth in a dose- and time-dependent manner, along with induction of G₀/G₁ arrest and apoptosis in SW480 cells.

Multiple molecular mechanisms seem to be involved in the tumor suppressive effect of CAPE. Recently, it was reported that CAPE could inhibit NF κ B, inducing apoptosis *via* Fas signal activation in human tumor cells^[11,12]. Hung *et al*^[22] reported that down-regulation of Mcl-1 gene expression and activation of caspase-8 are associated with CAPE-triggered cell apoptosis. Moreover, tumor suppressor proteins P53 and p38 MAPK play a prominent role in the CAPE-induced apoptotic cell death in C6 glioma cells, which might contribute to the anti-tumor effect in these cells^[13].

Our results suggest that the anti-tumor effects of CAPE on SW480 cells are associated with down-regulation of the β -catenin associated signaling pathways. β -catenin plays a dual role in cells. It is a structural component of cell-cell adherent junctions as well as a key player in β -catenin associated signaling pathway^[23]. The signaling function of β -catenin is particularly important in colorectal cancer since mutations of APC or tumor-associated mutations of β -catenin lead to its stabilization^[24,25]. Here we showed that CAPE treatment inhibited β -catenin in a dose-dependent manner in SW480 cells. More interestingly, CAPE changed the localization of β -catenin in cells. As shown in Figure 7, nuclear accumulated β -catenin in SW480 cells mainly relocalized on the cell membrane at cell-cell contacts after CAPE treatment. Two of target genes of the activated β -catenin associated signaling pathways have been reported to be c-myc and cyclin D1^[26,27]. Oncogenes c-myc and cyclinD1 are a kind of effector protein of the karyomitosis signal, which can trigger and regulate the transcription of the genes related with pro-

liferation. They are frequently overexpressed in several human tumors, including colorectal cancer. Our results showed that CAPE treatment markedly decreased the expression of c-myc and cyclinD1. This indicates that CAPE induced cell cycle arrest and apoptosis might be associated with down-regulation of β -catenin associated signaling pathways.

In summary, we have shown that CAPE can inhibit the growth of human SW480 colorectal cancer cells by inducing cell cycle arrest and apoptosis. Decreased β -catenin and the β -catenin associated signaling pathway target gene expression might mediate the anti-tumor effects of CAPE. These findings have strong implications for CAPE as a potential therapeutic agent for colorectal cancer.

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Gene expression analysis of primary normal human hepatocytes infected with human hepatitis B virus

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Abstract

AIM: To find the relationship between hepatitis B virus (HBV) and hepatocytes during the initial state of infection by cDNA microarray.

METHODS: Primary normal human hepatocytes (PNHHs) were isolated and infected with HBV. From the PNHHs, RNA was isolated and inverted into complement DNA (cDNA) with Cy3- or Cy5- labeled dUTP for microarray analysis. The labeled cDNA was hybridized with microarray chip, including 4224 cDNAs. From the image of the microarray, expression profiles were produced and some of them were confirmed by RT-PCR, immunoblot analysis, and NF- κ B luciferase reporter assay.

RESULTS: From the cDNA microarray, we obtained 98 differentially regulated genes. Of the 98 genes, 53 were up regulated and 45 down regulated. Interestingly, in the up regulated genes, we found the TNF signaling pathway-related genes: LT- α , TRAF2, and NIK. By using RT-PCR, we confirmed the up-regulation of these genes in HepG2, Huh7, and Chang liver cells, which were transfected with pHBV1.2 \times , a plasmid encoding all HBV messages. Moreover, these three genes participated in HBV-mediated NF- κ B activation.

CONCLUSION: During the initial state of HBV infection, hepatocytes facilitate the activation of NF- κ B through up regulation of LT- α , TRAF2, and NIK.

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Key words: cDNA microarray; Primary normal human hepatocytes; LT- α ; TRAF2; NIK; NF- κ B

INTRODUCTION

Human hepatitis B virus (HBV) is a causative agent for liver diseases such as cirrhosis and hepatocellular carcinoma (HCC)^[1]. Chronic infection of HBV affects approximately 800 million people and is the principal cause of chronic liver diseases^[2]. Moreover, HBV carriers have a much higher frequency of developing liver cancer than uninfected people^[3].

HBV has a small, partially double-stranded DNA genome. After viral infection of hepatocytes, the partially double-stranded DNA genome converts into covalently closed circular DNA (cccDNA) in nuclei^[4-7]. Several kinds of viral transcripts are then produced by the host RNA polymerase. The transcripts encode for viral polymerase, viral oncogene HBx protein, and viral structural proteins such as surface proteins and core proteins^[3].

Many efforts have been made to investigate the process of liver disease by HBV. Traditional techniques such as Northern blot and reverse transcription polymerase chain reaction (RT-PCR) for identification of genes differentially expressed by HBV infection have shown limited success, because only one gene or at best a handful of genes can be studied in one experiment. However, complementary DNA (cDNA) microarray allows the study of several thousands of genes at one time. To evaluate the relationship between HBV infection and liver diseases, recent studies have analyzed the gene expression profiles at tissue level. In these studies, the effects of HBV infection are analyzed by cDNA microarray analysis of HCC tissue samples^[8-10]. Through the analyses, many differentially expressed genes can be identified^[11]. The analyses, however, have mainly focused on the gene expression profiles of already transformed cells or long-term infected HBV hepatocytes. Therefore, these analyses mostly stem from the analysis of the end result of pathogenesis of HBV in hepatocytes rather than the analysis of ongoing pathogenesis of HBV infection in hepatocytes. In this report, however, we focused on the gene expression profile analysis of the early stage HBV infection, thereby excluding factors such as responses to host immune surveillance. To mimic the early

stage HBV infection of hepatocytes, we isolated primary normal human hepatocytes (PNHHs) and the cells were infected with HBV in culture. These conditions were chosen as they could represent the most similar conditions to those *in vivo*, except for the absence of other types of cells such as immunocytes. Therefore, gene expression profiles in this report could show the result of interaction only between HBV and PNHHs. In this study we have identified 45 down-regulated genes and 53 up-regulated genes.

MATERIALS AND METHODS

Construction

pHBV1.2 \times , a plasmid which provides all HBV transcripts, was used to infect PNHHs as previously described^[12]. This construct was similar to that as described by Guidotti *et al.*^[13]. Mammalian expression vector for NIK and NIK DN (aa 624-947) was provided by DV Goeddel (Turarik Inc.)^[14], for TRAF2 and TRAF2 DN (aa 241-501) by Dr. SY Lee^[15].

HBV production and infection

We transfected HepG2 cells with pHBV1.2 \times constructs for generation of HBV using Fugene 6 transfection reagent (Roche) as instructed by the manufacturer. After transfection, the cells were cultured for 5 d and harvested. HBV particles in the harvested media were cleared and concentrated through ultracentrifugation using PST55Ti rotor (Hitachi) for 1 h at 220 000 g with 3 mL cushion buffer containing 20 g/L sucrose, 50 mmol/L Tris-HCl pH 7.5, and 30 mmol/L NaCl. After ultracentrifugation, the pellet was resuspended with 1 \times PBS. The resuspended viral solution was filtered with a 0.2 μ m pore filter (Millipore). The titer of HBV solution was adjusted to 10⁹ virus genome equivalent (v.g.e.) per mL. PNHHs were infected with the above virus solution at about 100 v.g.e. per cell. Using this method, the efficiency of HBV infection to PNHHs was generally 50%^[16].

Isolation and culture of PNHHs

Healthy parts of a liver from a patient who underwent hepatic resection for an intrahepatic stone at the Inje University Paik Hospital, Pusan, Korea was obtained and used as the source of hepatocytes. The removed tissue was immediately placed in Hank's balanced salt solution (HBSS) and processed for cell culture. Isolation of hepatocytes was performed using a two-step collagenase perfusion technique^[17,18]. The isolated hepatocytes were resuspended in a nutrient medium containing 90 mL/L Williams' E and 10 mL/L Medium 199, supplemented with 10 μ g/mL insulin, 5 μ g/mL transferrin, 10⁻⁷ mol/L sodium selenite, and 50 mL/L FBS.

Confirmation of PNHH infection with HBV

A test was performed with the isolated DNA to determine whether the HBV-infected PNHHs formed cccDNA. Amplification with primers specific for both outside regions was performed with the isolated DNA because the region was specific for cccDNA rather than partially double-stranded DNA found in viral particles. The primers

used are (5'-CTATGCTGGGTCTTCCAAATT-3') which anneals to near the codon for amino acid 80 in human HBc open reading frame (ORF) and (5'-TTTCTGTGTA-AACAATATCTG-3') which anneals to near the codon for amino acid 680 in HBV Pol ORF. Therefore, if cccDNA was present in the isolated DNA, an amplified 1 kb product could be obtained. In this test, DNA isolated from mock-infected PNHHs was used as the negative control. A PCR test was performed with RNA to confirm whether HBV transcripts were produced. Reverse transcription amplification was performed with primers specific for the epsilon regions: (5'-CAACTTTTTTTCACCTCTGCC-TA-3') which anneals to DR1 and the reverse primer (5'-GATCTCGTACTGAAAGGAAAGA-3'). In addition, to detect HBV genome, real-time PCR was also performed as previously described^[12].

cDNA array analysis

RNA was isolated using TRIzol reagent (Life Technologies) according to the manufacturer's instructions. With the total isolated RNA, reverse transcription was performed with Cy5-labeled dUTP in the experimental sample and Cy3-labeled dUTP in the control. Fifty micrograms of RNA, 1.5 ng oligo dT primer, and 1 ng control RNA containing lambda DNA sequences with a poly A sequence at the 3' ends for reverse transcription, were mixed and volume of the mixture was adjusted to 20 μ L. The mixture was incubated at 70°C for 5 min. After incubation, the mixture was quickly cooled on ice. With this whole reaction mixture, the labeling reaction was performed under the following conditions: 1 \times reverse transcription buffer, 0.6 mmol/L Cy3- or Cy5-dUTP, 40 U of RNase inhibitor (Roche), 50 U of AMV-RT (Roche) and a dNTP mix containing 1 mmol/L dATP, 1 mmol/L dGTP, 1 mmol/L dCTP, and 0.4 mmol/L dTTP at 42°C for 1 h. After 1 h, 50 U of AMV-RT was added to the reaction mixture and the mixture was further incubated for 1 h for complete reverse transcription. Reverse transcription was stopped by the addition of 5 μ L 0.5 mol/L EDTA. The synthesized cDNA was purified using a chromaspin column (Clontech) as instructed by the manufacturer and precipitated with ethanol. Both Cy3- and Cy5- labeled cDNAs were resuspended with 100 μ L hybridization buffer, containing 6 \times standard saline citrate (SSC), 2 g/L sodium dodecyl sulfate (SDS), 5 \times Denhardt solution, and 1 mg/mL salmon sperm DNA. The labeled cDNA was used for hybridization to the cDNA microarray chip at 62°C. The chip was arrayed using a GMS417 arrayer (Genetic MicroSystems Inc., Woburn, MA) with 4224 cDNAs and internal standards such as tubulin and actin and external standards such as lambda DNA. After 16-18 h of hybridization, the hybridized array was washed twice at 58°C for 30 min with washing buffer I containing 2 \times SSC and 2 g/L SDS and washed once with washing buffer II containing 0.05 \times SSC at room temperature for 5 min.

Analysis of chips

For quantification of the signals, the chips were scanned using an array scanner generation III (Molecular Dynamics) followed by image analysis using ImaGene ver. 3.0 software (BioDiscovery Ltd., Swansea, UK). The signal intensity

of each spot was adjusted to obtain more accurate data by subtracting the background signals from the immediate surroundings. In this analysis, a difference in the ratio of more than two folds was considered significant.

Cells and transfection

HepG2, Huh7, and Chang liver cells were maintained in minimum essential media (Sigma) supplemented with 100 mL/L fetal bovine serum. For reverse transcription-polymerase chain reaction and luciferase reporter assay, cells were seeded in 12- well plates at a density of 0.2×10^6 cells per well and transfected on the following day with the appropriate DNA and fugene 6 (Roche) as described by the manufacturer. To normalize the total DNA, pUC119 and backbone DNA of pHBV1.2x were used. The transfection efficiency for HepG2 with fugene 6 was usually 10%-20%.

RT-PCR analysis

Cells were transfected with pUC119 and pHBV1.2x. After 48 h of transfection, total RNA was extracted with TRIzol reagent (Life Technologies) as described by the manufacturer. cDNA was produced by reverse transcription using the same procedure as cDNA microarray analysis. Following reverse transcription, the synthesized cDNA was amplified with 2.5 U Hot start Taq polymerase (Takara), GAPDH specific primer set, and appropriate primer set. The sequences of the primer set are as follows: TRAF2 specific forward(5'-AGGGGACCCT-GAAAGAATAC-3'), TRAF2 specific reverse(5'-CAGGGCTTCAATCTTGTCTT-3'), NIK specific forward(5'-TACCTCCACTCACGAAGGAT-3'), NIK specific reverse(5'-CAAGGGAGGAGACTTGTTTG-3'), LT- α specific forward(5'-AGCTATCCACCCACACAGAT-3'), LT- α specific reverse(5'-GTTTATTGGGCTTCATC-GAG-3'), GAPDH specific forward(5'-ATCATCCCTGCCTCTACTGG-3'), and GAPDH specific reverse (5'-TGGGTGTCGCTGTTGAAGTC-3'). PCR amplification was performed using Gene mp PCR system 2400 (Perkin-Elmer) with 5 min initial denaturation at 95°C and 35 cycles of 50 s at 95°C, 50 s at 60°C, and 50 s at 72°C, followed by 7 min of extension at 72°C. To separate the PCR fragments, 15% agarose gel was used.

Immunoblot analysis

To confirm NIK and TRAF2 protein expression, we performed immunoblot analysis with anti-NIK rabbit polyclonal antibody (Santa Cruz) and anti-TRAF2 mouse monoclonal antibody (Santa Cruz). HepG2 cells were seeded in 6- well plates at a density of 0.4×10^6 cells per well. Cells were lysed with RIPA buffer containing 25 mmol/L Tris-HCl (pH 7.4), 150 mmol/L KCl, 5 mmol/L EDTA, 10 mL/L Nonidet P-40 (NP-40), 5 g/L sodium deoxycholate, and 1 g/L SDS and centrifuged at 12000 g for 10 min. The supernatants were separated by 12% SDS-PAGE protein gel for immunoblot analysis.

Luciferase reporter assay

After seeded on 12-well plates, HepG2 cells were co-transfected with appropriate DNA (Figure 5), 0.1 μ g pNF- κ B-

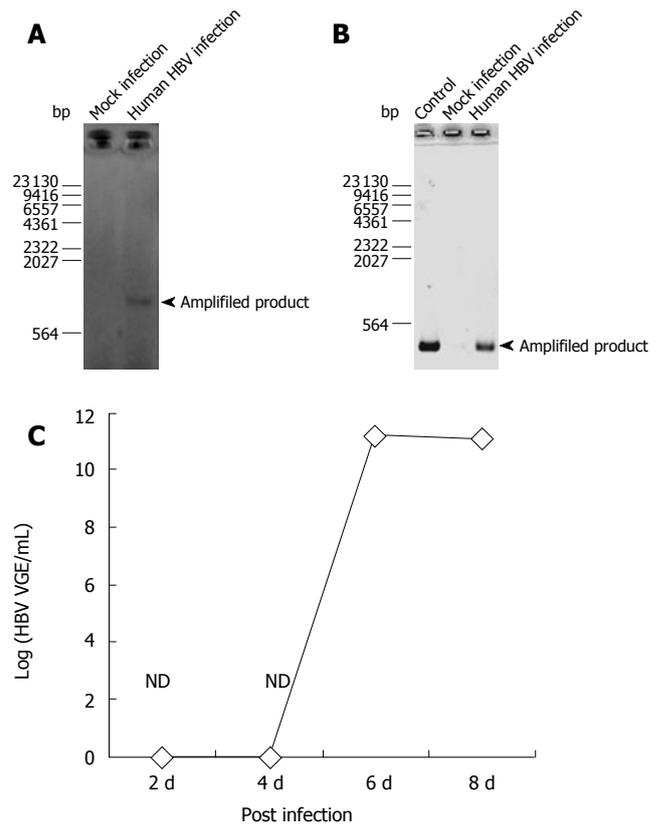


Figure 1 Confirmation of HBV infection for PNHHs using PCR for cccDNA formation and RT-PCR for production of transcripts. From PNHHs infected with HBV, RNA and DNA were extracted using TRIzol reagent as described in Materials and Methods. As a negative control, mock-infected PNHHs were used. **A:** With the extracted DNA, cccDNA was confirmed by a PCR analysis as described in Materials and Methods; **B:** With the extracted RNA, a transcript of HBV was confirmed by RT-PCR analysis as described in Materials and Methods; **C:** With the extracted RNA, a transcript of HBV was confirmed by real-time PCR as previously described^[19].

luciferase and 0.1 μ g pCMV- β -galactosidase. After 48 h of transfection, cell extracts were prepared and luciferase reporter assay was performed using a luciferase assay system (Promega) as described by the manufacturer. The transfection efficiency was normalized by its galactosidase activity. The assay was triplicated and repeated at least twice.

RESULTS

Confirmation of PNHH infection with HBV

To confirm HBV infection to PNHHs, DNA was isolated during the RNA purification step with TRIzol reagent as instructed by the manufacturer. Infection was confirmed through PCR-based amplification specific only for cccDNA in nuclei of the infected cells. Figure 1 shows that the amplified product appeared in HBV- infected cells, indicating that HBV did infect PNHHs and that the nucleocapsid was transported into nuclei of the infected hepatocytes (Figure 1A). RT-PCR analysis of the HBV transcripts amplified with primers specific for epsilon and polymerase regions as described in Materials and Methods confirmed the presence of HBV RNA transcripts in the infected cells (Figure 1B). Real time PCR showed that HBV was not detected until 6 d after infection (Figure 1C).

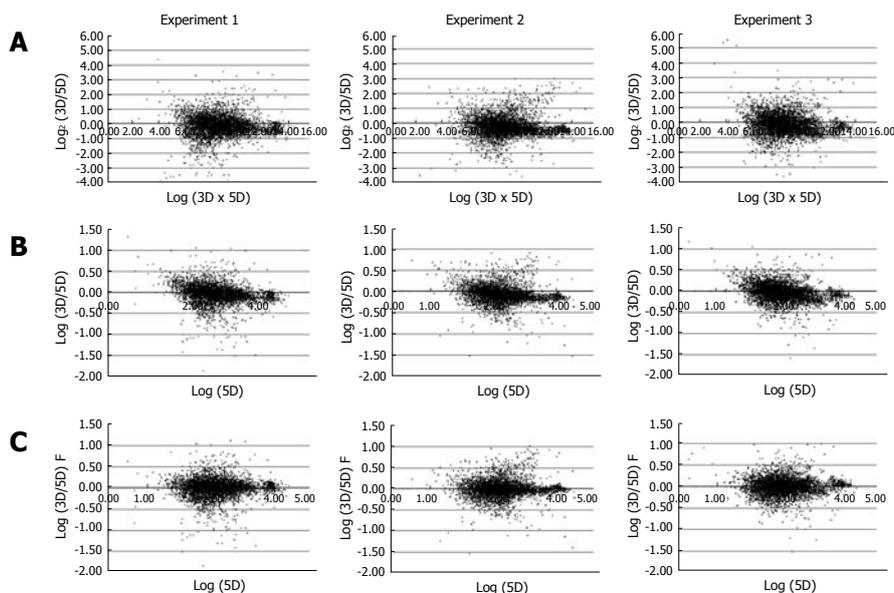


Figure 2 Scatter plot analysis. For normalization of the Cy3 (3D) and Cy5 channel signal (5D) channels, data obtained from the raw image scanning were plotted in a scatter plot using Excel software (Microsoft). **A:** The X-axis represents $\text{Log}_2(3D/5D)$ and the Y-axis $\text{Log}_2(3D/5D)$; **B:** The X-axis represents $\text{Log}(5D)$ and the Y-axis $\text{Log}(3D/5D)$; **C:** The X-axis represents $\text{Log}(5D)$ and the Y-axis $\text{Log}(3D/5D) F$, in which "F" is the function for normalization. The bottom panel shows data with signals fitted to an exponential decay curve.

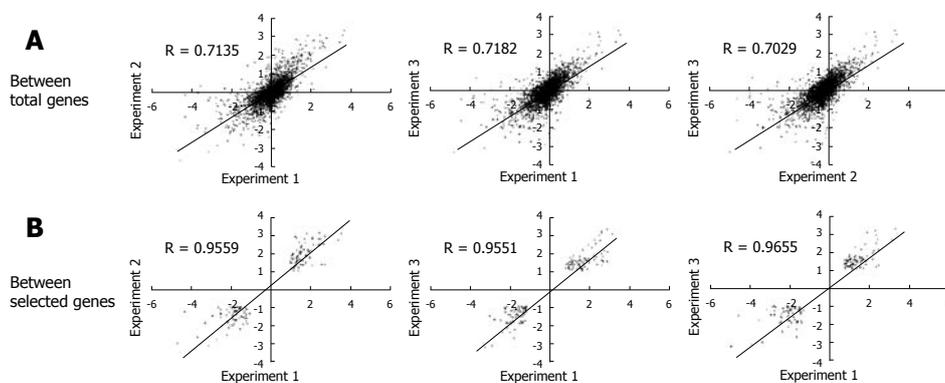


Figure 3 Correlation between three sets of PNHHs infected for eight days. **A:** With the reliable signals in obtained signals, the correlation efficient was calculated between each experiment; **B:** In addition, another correlation efficient was also calculated with only the selected genes, which were differentially expressed more than 2 folds.

Raw data analysis

The experiments were carried out in triplicate at the infection step for more certain identification of genes differentially expressed by HBV infection. From each of the HBV infected cells for over 8 d, RNA was isolated and analyzed by microarray. As a result, three sets of cDNA array images were obtained. We analyzed the intensity of the raw image through scatterplot analyses. Figure 2A shows scatterplot analyses of $\log(\text{Cy3 signal} \times \text{Cy5 signal})$ *vs* $\log_2(\text{Cy3 signal}/\text{Cy5 signal})$. This showed that each plot tended to divert from the general small curve (Figure 2A). But, each scatterplot analysis of $\text{Log}(\text{Cy3 signal}/\text{Cy5 signal})$ *vs* $\text{Log}(\text{Cy5 signal})$ showed a curve closer to the exponential decay (Figure 2B). Therefore, the data were fitted to an exponential decay curve for Cy3 per Cy5 channel correction (Figure 2C). Through these steps, we obtained a higher confidence ratio of the Cy3 signal compared to the Cy5 signal for each chip. With the ratios obtained, we analyzed the correlation coefficient between the data of the three chips. The correlation coefficient turned out to be more than 0.7 (Figure 3A), suggesting that the relationship between each chip was significant. The correlation coefficient for genes that were differentially expressed more than two folds was more than 0.95 (Figure 3B). Selected genes that were differentially expressed more than two folds, showed a high reproducibility among the triplicate microarray

analyses.

Analysis of differentially regulated genes

Through a microarray analysis of PNHHs infected with HBV, we obtained the profile of 45 genes that were down regulated more than two folds compared to the control. The 45 down-regulated genes were analyzed classified by function (Table 1). Table 1 shows that many transcription factors related to RNA polymerase II, were down-regulated by HBV infection. In contrast, transcription factors such as C/EBP, which is used for transcription of HBV genes^[19,20], were not differentially expressed. That is, the C/EBP expression level was changed less than two folds.

From the analysis by cDNA microarray, 53 up-regulated genes were identified by an increase of more than two folds in their differential expression. Table 2 shows that growth- and tumor-related molecules comprised a proportion of the up-regulated genes. The positive effector genes for tumor and proliferation have found to be GDF11^[21] and NOL1^[22] and the negative effector genes EXTL3^[23] and RAD50. The most interesting genes have found to be the TNF signaling pathway- related genes. LT- α is an inflammatory cytokine and induces the TNF signaling pathway as a ligand for TNF receptor (TNFR). LT- α binds to TNFR and recruits TRAF2. MAP3K14 (NF- κ B inducing kinase, NIK) binds to TRAF2 and activates NF- κ B^[24].

Table 1 Forty-five differentially down-regulated genes obtained and categorized by their function

Category	UniGene	Gene name	Symbol	Locus	Function	Control/ HBV infection	P-value
Transcription/ RNA Pol II	Hs.442675	Thyroid hormone receptor interactor 8	TRIP8	10	Transcription co-activator of Pol II promoter	3.469	0.030
Transcription	Hs.57475	Sex comb on midleg homolog 1	SCMH1	1p34	Pol II transcription	3.315	0.019
Transcription	Hs.119014	Zinc finger protein 175	ZNF175	19q13.4	C2H2 zinc-finger protein 175	2.942	0.017
Transcription/ RNA Pol II	Hs.437905	Spi-B transcription factor (Spi-1/PU.1 related)	SPIB	19q13.3-q13.4	RNA polymerase II transcription factor	2.743	0.017
Transcription/ RNA Pol II	Hs.148427	LIM homeobox protein 3	LHX3	9q34.3	RNA Pol 2 transcription factor and activate pituitary hormone genes	2.492	0.006
Signal	Hs.17154	Dual-specificity tyrosine-(Y)- phosphorylation regulated kinase 4	DYRK4	12p13.32	Dual-specificity protein kinase 4	3.938	0.005
Signal	Hs.262886	Inositol polyphosphate-5- phosphatase, 145kD	INPP5D	2q36-q37	Modulating cytokine signaling within the hemopoietic system	3.587	0.009
Signal	Hs.75249	ADP-ribosylation factor-like 6 interacting protein	ARL6IP	16p12-p11.2	Activator of phospholipase D (PLD)	2.801	0.010
Tumor/Suppress	Hs.77793	c-src tyrosine kinase	CSK	15q23-q25	Downregulate the tyrosine kinase activity of the c-src oncoprotein	3.377	0.021
Tumor/Induce	Hs.89839	EphA1	EPHA1	7q34	Overexpression of EPH mRNA was found in a hepatoma	3.039	0.022
Tumor/Induce	Hs.79070	V-myc avian myelocytomatosis viral oncogene homolog	MYC	8q24.12-q24.13	Promotes cell proliferation and transformation	2.357	0.011
Immune response	Hs.118354	Human MHC Class I region proline rich protein mRNA	CAT56	6p21.32	Immune response	2.588	0.024
Miscellaneous	Hs.180610	Splicing factor proline/ glutamine rich	SFPQ	1p34.3	Pre-mRNA splicing factor required for pre-mRNA splicing	10.471	0.006
Miscellaneous/ Cytoskeleton	Hs.75064	Tubulin-specific chaperone c	TBCC	6pter-p12.1	Cofactor in the folding pathway of beta-tubulin	10.2	0.020
Miscellaneous	Hs.438683	BCM-like membrane protein precursor	SBB142	1q23.1	BCM-like membrane protein precursor	3.751	0.004
Miscellaneous	Hs.8203	Endomembrane protein emp70 precursor isolog	LOC56889	10q24.2	Low similarity to human endosomal protein P76	3.502	0.038
Miscellaneous	Hs.311609	Nuclear RNA helicase, DECD variant of DEAD box family	DDXL	19p13.13	Member of the DEAD/H box ATP- dependent RNA helicase family	2.868	0.026
Miscellaneous/ Energy	Hs.150922	BCS1 (yeast homolog)-like	BCS1L	2q33	Function in the assembly of complex III of the respiratory chain	2.682	0.007
Miscellaneous	Hs.6679	hHDC for homolog of Drosophila headcase	LOC51696	6q23-q24	hHDC for homolog of Drosophila headcase	2.611	0.020
Miscellaneous	Hs.5300	Bladder cancer associated protein	BLCAP	20q11.2-q12	Appears to be down-regulated during bladder cancer progression	2.459	0.028
Miscellaneous	Hs.179526	Upregulated by 1, 25-dihydroxyvitamin D-3	VDUP1	1q21.2	Upregulated by 1, 25-dihydroxyvitamin D-3	2.394	0.043
Miscellaneous	Hs.440961	Calpastatin	CAST	5q15-q21	Inhibitor of the cysteine (thiol) protease calpain	2.276	0.000
Miscellaneous	Hs.275775	Selenoprotein P, plasma, 1	SEPP1	5q31	An oxidant defense in the extracellular space	2.186	0.007
EST	Hs.371233	ESTs		Xp22.3	Moderately similar to T08795 hypothetical protein DKFZp586J1822.1	7.826	0.025
EST	Hs.229338	ESTs		X		4.687	0.007
EST	Hs.212957	ESTs		3q26.1	Moderately similar to ZN91_HUMAN ZINC FINGER PROTEIN 91	4.611	0.019
EST	Hs.211823	ESTs		2q37.1		4.519	0.030
EST	Hs.57836	ESTs		17		3.323	0.029
EST	Hs.87912	ESTs		14q24.1		3.314	0.013
EST	Hs.12429	ESTs	FLJ22479	4q26-q27	Hypothetical protein FLJ22479	3.217	0.041
EST	Hs.213586	ESTs		7		2.759	0.044
EST	Hs.2755711	ESTs		22	Weakly similar to T20379 hypothetical protein	2.723	0.019
EST	Hs.191435	ESTs		8p23.1-p22	Weakly similar to S65657 alpha-1C- adrenergic receptor splice form 2	2.638	0.023
EST	Hs.31293	ESTs		9p13.1		2.286	0.035
Predicted protein	Hs.414464	Hypothetical protein	HSD3.1	14q31.3		7.314	0.008

Predicted protein	Hs.100914	Hypothetical protein FLJ10352	FLJ10352	18p11.21		6.239	0.006
Predicted protein	Hs.181112	HSPC126 protein	HSPC126	13q14.12		3.322	0.045
Predicted protein	Hs.306711	KIAA1081 protein	ELKS	12p13.3		3.122	0.036
Predicted protein	Hs.101891	KIAA1193 protein	KIAA1193	19p13.3	Weakly similar to RPB1_HUMAN DNA-directed RNA Pol II largest subunit	3.029	0.030
Predicted protein	Hs.272759	KIAA1457 protein	KIAA1457	12q24.31		2.98	0.020
Predicted protein	Hs.172089	Homo sapiens mRNA; cDNA DKFZp58612022		11q22.1		2.784	0.036
Predicted protein	Hs.7049	Hypothetical protein FLJ11305	FLJ11305	13q34		2.65	0.028
Predicted protein	Hs.445255	KIAA0368 protein	KIAA0368	9q32		2.423	0.016
Predicted protein	Hs.192190	KIAA0782 protein	KIAA0782	11q13.3		2.332	0.009
Predicted protein	Hs.169910	KIAA0173 gene product	KIAA0173	2p24.3-p24.1	Similar to S72482 hypothetical protein	2.171	0.014

Table 2 Fifty-three differentially up-regulated genes obtained and categorized by their function

Category	UniGene	Gene name	Symbol	Locus	Function	Control/HBV	P-value infection
Signal	Hs.82887	Protein phosphatase 1, regulatory (inhibitor) subunit 11	PPP1R11	6p21.3	Soluble protein phosphatase inhibitor(repressor)	3.623	0.026
Signal	Hs.437575	TNF receptor-associated factor 2	TRAF2	9q34	Required for activation of NFkappaB	3.23	0.026
Signal	Hs.6527	G protein-coupled receptor 56	GPR56	16q13	Member of the G protein-coupled receptor family	2.817	0.009
Signal	Hs.29203	Homo sapiens G protein beta subunit mRNA, partial cds	GBL	16p13.3	G protein-linked receptor protein for signalling pathway	2.76	0.009
Signal/ Cytoskeleton	Hs.2157	Wiskott-Aldrich syndrome	WAS	Xp11.4-p11.21	Involved in transduction of signals from receptors on the cell surface to the actin cytoskeleton	2.446	0.018
Signal	Hs.440315	Mitogen-activated protein kinase kinase kinase 14	MAP3K14	17q21	Binds to TRAF2 and stimulates NF-kappaB activity	2.185	0.043
Tumor/ Induce	Hs.15243	Nucleolar protein 1 (120kD)	NOL1	12p13.3	Transforms NIH3T3 cells when overexpressed	5.273	0.004
Tumor/ Supress	Hs.9018	Exostoses (multiple)-like 3	EXTL3	8p21	Tumor suppressor, glycosyltransferase activity	5.07	0.093
Tumor/ Supress	Hs.41587	RAD50 (S. cerevisiae) homolog	RAD50	5q31	Associates with MRE11, nibrin (NBS1) and the tumor suppressor BRAC1	2.443	0.013
Growth/ Positive	Hs.511740	Growth differentiation factor 11	GDF11	12q13.13	Regulators of cell growth and differentiation in both embryonic and adult tissues	2.969	0.023
Cell cycle/ Negative	Hs.76364	Allograft inflammatory factor 1	AIF1	6p21.3	Involved in negative regulation of growth of vascular smooth muscle cells	3.573	0.031
Cell cycle/ Positive	Hs.25313	Microspherule protein 1	MCRS1	12q13.12	Involved in cell-cycle-dependent stabilization of ICP22 in HSV1-infected cells	3.273	0.044
Cell cycle/ Positive	Hs.371833	Nuclear receptor binding factor-2	NRBF-2	10	A possible gene activator protein interacting with nuclear hormone receptors	2.568	0.018
Cell cycle/ Positive	Hs.440606	Centrosomal protein 2	CEP2	20q11.22-q12	Regulate centriole-centriole cohesion during the cell cycle	2.422	0.022
Enzyme/ Glycosylation	Hs.4814	Mannosidase, alpha, class 1B, member 1	MAN1B1	9q34	N-linked glycosylation	3.097	0.008
Enzyme/ lysophospholipase	Hs.889	Charot-Leyden crystal protein	CLC	19q13.1	Phospholipid metabolism and anti-pathogen	3.065	0.035
Enzyme/Protease	Hs.75890	Site-1 protease	MBTPS1	16q24	A sterol-regulated subtilisin-like serine protease	2.873	0.023
Immune response	Hs.2014	T cell receptor delta locus	TRD@	14q11.2	T-cell antigen receptor, delta polypeptide	3.69	0.023
Immune response	Hs.465511	Granzyme M	GZMM	19p13.3	Serine protease for anti-pathogen response	3.201	0.023

Transcription	Hs.436871	Zinc finger protein 173	ZNF173	6p21.3	DNA/protein binding, transcriptional protein	3.411	0.002
Transcription	Hs.108139	Zinc finger protein 212	ZNF212	7q36.1	DNA/protein binding, transcriptional protein	2.341	0.045
Apoptosis	Hs.36	Lymphotoxin alpha	LTA	6p21.3	A member of the tumor necrosis factor family	14.912	0.019
Miscellaneous	Hs.434384	Titin	TTN	2q31	Large myofilament protein	4.087	0.012
Miscellaneous	Hs.58927	Nuclear VCP-like	NVL	1q41-q42.2	Member of the AAA family of ATPases	4.005	0.035
Miscellaneous	Hs.122552	G-2 and S-phase expressed 1	GTSE1	22q13.2-q13.3	Accumulates in late S/G2 phase, is phosphorylated in mitosis, and disappears in G1 phase	3.491	0.007
Miscellaneous/ Glycosylation	Hs.82921	Solute carrier family 35 (CMP-sialic acid transporter), member 1	SLC35A1	6q15	Important for normal sialylation of glycoproteins and glycolipids	3.352	0.013
Miscellaneous	Hs.410455	Unc119 (C.elegans) homolog	UNC119	17q11.2	May function in photoreceptor neurotransmission	3.328	0.030
Miscellaneous	Hs.55041	CGI-22 protein	MRPL2	6p21.3	Unknown	3.052	0.01
Miscellaneous/ Cytoskeleton	Hs.74088	Bridging integrator-3	BIN3	8q21.2	Related to actin assembly-competent state	2.677	0.018
Miscellaneous	Hs.25237	Mesenchymal stem cell protein DSCD75	LOC51337	8q24.3	Moderately similar to uncharacterized Drosophila CG4666	2.615	0.042
EST	Hs.95867	Homo sapiens EST00098 gene, last exon	EST00098	9q34.1		8.781	0.027
EST	Hs.98785	ESTs	KSP37	4p16		3.749	0.011
EST	Hs.136912	ESTs	MGC10796	3q13.13		3.435	0.003
EST	Hs.101774	ESTs	FLJ23045	20p11.23		3.387	0.032
EST	Hs.420262	ESTs		13		3.355	0.022
EST	Hs.124840	ESTs		11q13.1		3.114	0.021
EST	Hs.272299	ESTs	RP4-622L5	1p36.11-p34.2		3.008	0.034
EST	Hs.415048	ESTs		5		2.891	0.025
EST	Hs.531268	ESTs		16		2.889	0.013
EST	Hs.273830	ESTs	FLJ12742	1		2.739	0.020
EST	Hs.190162	ESTs		1p32.3		2.708	0.021
EST	Hs.303172	ESTs		18		2.578	0.04
EST	Hs.59203	ESTs		7		2.437	0.022
EST	Hs.231444	ESTs		1		2.343	0.005
Unknown sequence	Hs.284265	Homo sapiens pRGR1 mRNA, partial cds		6q27		2.966	0.043
Unknown sequence	Hs.291385	Homo sapiens clone 23664 and 23905 mRNA sequence		4p14-p12		2.439	0.041
Predicted protein	Hs.31718	Homo sapiens cDNA FLJ11034 fis, clone PLACE1004258	VRL			17.359	0.000
Predicted protein	Hs.61960	Hypothetical protein	FLJ20040	16p13.3		9.166	0.002
Predicted protein	Hs.274552	Homo sapiens cDNA FLJ10720 fis, clone NT2RP3001116	FLJ10720	5		4.751	0.015
Predicted protein	Hs.279761	HSPC134 protein	HSPC134	14q11.2		3.501	0.038
Predicted protein	Hs.283716	Hypothetical protein PRO1584	PRO1584	8p21.2		3.493	0.033
Predicted protein	Hs.464526	Homo sapiens clone 23649 and 23755 unknown mRNA, partial cds		18q11.2		3.198	0.032
Predicted protein	Hs.274412	Homo sapiens cDNA FLJ10207 UPF3A fis, clone HEMBA1005475		17p11.2		3.076	0.012

LT- α , TRAF2, and NIK were also up-regulated in the experiment (Table 2). This means that HBV activates NF- κ B through up-regulation of LT- α , TRAF2, and NIK.

RT-PCR analysis and immunoblot assay of selected genes

According to the cDNA microarray data, three genes related to the TNF signaling pathway, LT- α , TRAF2, and NIK, were up-regulated. Upregulation of these genes was confirmed by RT-PCR. For RT-PCR analysis, primer sets

specific to LT- α , TRAF2, and NIK, were used and experiments were performed in hepatoma-derived cell lines, including HepG2, Huh7, and Chang liver cells. As a result, the mRNA levels of these three genes in each cell line were increased by pHBV1.2 \times transfection (Figure 4). In addition to RT-PCR, the expression of NIK and TRAF2 was confirmed at the protein level (Figure 5). The expression of LT- α , was confirmed by immunofluorescence staining analysis (data not shown).

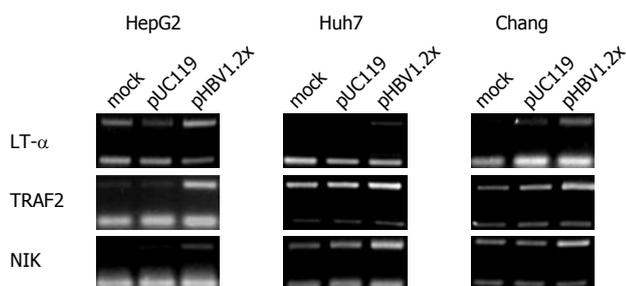


Figure 4 RT-PCR analysis of selected genes. Mock means untransfected cells. pUC119 is backbone of pHBV1.2x, so pUC119 transfected cells are the negative control for pHBV1.2x transfected cell. In the hepatoma-derived cell lines, HepG2, Huh7, and Chang liver cells, TRAF2, NIK, and LT- α mRNA level in pHBV1.2x transfected cells were up-regulated rather than mock and pUC119 transfected cells.

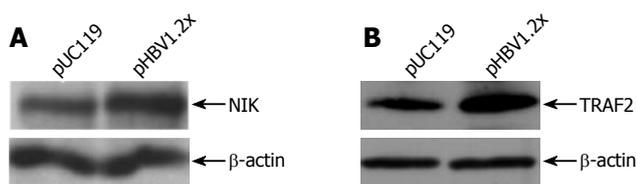


Figure 5 Immunoblot assay of TRAF2 and NIK. pUC119 is a backbone DNA about pHBV1.2x. In the HepG2 cells transfected with pHBV1.2x, the protein levels of NIK (A) and TRAF2 (B) were increased. β -actin was used to normalize total protein level.

NF- κ B activation through TRAF2, NIK mRNA up regulation

According to cDNA microarray and RT-PCR analysis, mRNA expression of LT- α , TRAF2, and NIK was up-regulated by HBV. Since their expression was related to NF- κ B activation. HBV-mediated NF- κ B activation might be involved in the up regulation of these genes. To determine whether these genes actually are involved in HBV-mediated NF- κ B activation, we performed a luciferase assay with a pNF- κ B-luciferase vector as a reporter plasmid. To elucidate whether HBV-mediated NF- κ B activation is dependent on TRAF2 and NIK of three genes, we cotransfected pTRAF2 DN or pNIK DN, the dominant negative form of pTRAF2 or pNIK, with pNF- κ B-luciferase, pCMV β -galactosidase, and pHBV1.2x (Figure 6). The experiment for LT- α was performed with anti- LT- α , to neutralize LT- α . The pHBV1.2x produced about a 4.2 folds greater increase in NF- κ B luciferase activity than pUC119. However, pHBV1.2x cotransfection with TRAF2 DN or NIK DN produced about a 3.5 or 1.2 fold relative increase (Figure 6) and treatment with anti- LT- α decreased the ratio to less than 4.2 folds (data not shown). These findings led us to think that HBV could activate NF- κ B and that this HBV-mediated NF- κ B activation might require LT- α , TRAF2, and NIK. DC.

DISCUSSION

In this report, we focused on the interaction between HBV and hepatocytes during the initial stage of infection. To mimic hepatocyte infection with HBV under *in vivo* conditions, we isolated PNHHs and infected them with HBV. We chose this method because cultured cell lines

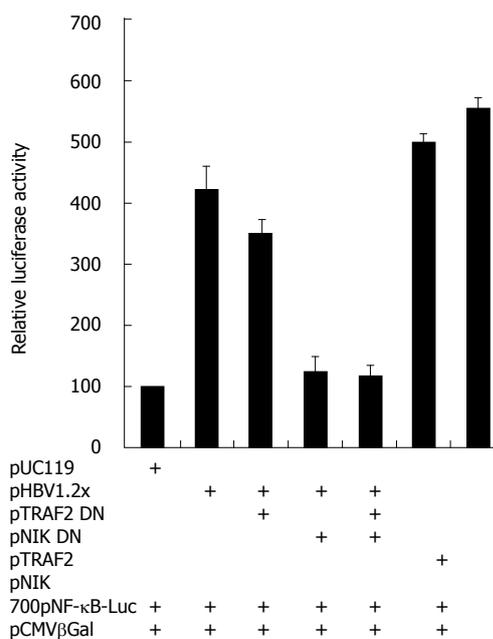


Figure 6 HBV-mediated NF- κ B activation through TRAF2 and NIK. In pHBV1.2x transfected HepG2 cells, NF- κ B activity was increased more than pUC119 transfected cells. But in cotransfected cells with pHBV1.2x and TRAF2 DN or NIK DN, NF- κ B activity was decreased less than pHBV1.2x transfected cells.

such as HepG2 are seldom infected with HBV^[25,26], and transformed cultured cells have many physiological properties that are altered in the original state of hepatocytes^[27,28]. In this experiment, the same hepatocytes were used as a control. Since they are produced under identical conditions, a pair of samples of the same genetic background could be obtained. With these samples, we were able to analyze differentially expressed genes. As a result, we obtained gene expression profiles and 98 consistently differentially expressed genes were identified by gene expression profiles. Of these genes, 53 were up-regulated and 45 down-regulated. It was reported that there are no genes uniformly correlated with HBV DNA profile during the initial host response to HBV infection^[29]. However, because this study was performed on chimpanzees, there are some considerations in making a comparison between this study with our report. Our report analyzed the effect of HBV on PNHHs at cellular level without any other cell types, including immunocytes. So the influence of immunocytes was not included in this analysis. In addition, the difference in human beings and chimpanzees needs to be taken into consideration.

The results of our study showed that a proportion of the down-regulated genes was transcription factor-related genes and a proportion of the up-regulated genes was TNF signaling pathway-related genes. Down regulation of transcription factors may be helpful for the transcription of the HBV gene because the transcripts of the host cell can be repressed and the transcriptional machinery can be efficiently used for viral transcription. C/EBP, which is involved in viral genome transcription^[19,20], had no substantial differential expression in this experiment. In addition to down regulation of transcription factor for virus transcription, up regulation of cell proliferation-related genes may help viral replication. Of the up-regulated genes,

LT- α , TRAF2, and NIK may induce cell proliferation *via* NF- κ B activation.

In fact, LT- α is mainly related to the signal cascade for apoptosis and generally involves the host defense system^[30]. However LT- α is also related to cell proliferation. Usually, TNF signaling including LT- α , can induce apoptosis and proliferation^[31]. TNF signaling by LT- α has a signal cascade from TNFR to TRADD. In the case of apoptosis, TRADD-FADD interaction is needed to activate caspase 8^[31]. In the case of proliferation, TRADD-TRAF2 interaction induces activation of NF- κ B, a proliferation-inducing transcription factor^[31]. After TRAF2 binds to TRADD, NIK binds to TRAF2 and activates NF- κ B through IKK activation and I κ B- α degradation^[24,32-34]. In cDNA microarray data, among genes related to the two opposite effects initiated by LT- α , proliferation-related genes are up-regulated. FADD is not differentially altered by more than two folds. Therefore, HBV infection may strengthen the TNF signaling pathway to cell proliferation through the induction of gene expression.

In conclusion, HBV induces NF- κ B activation by up-regulating LT- α , TRAF2, and NIK, and cell proliferation by activating NF- κ B.

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BASIC RESEARCH

Caffeic acid phenethyl ester modifies the Th1/Th2 balance in ileal mucosa after γ -irradiation in the rat by modulating the cytokine pattern

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Abstract

AIM: To pharmacologically modulate Th polarization in the ileum exposed to ionizing radiation by using the immuno-modulatory/apoptotic properties of Caffeic Acid Phenethyl Ester (CAPE).

METHODS: Rats received CAPE (30 mg/kg) treatment ip 15 min prior to intestinal 10 Gy γ -irradiation and once a day for a 6 d period after irradiation. Expression of genes implicated in Th differentiation in ileal mucosa (IL-23/IL-12R β 2), Th cytokine responses (IFN- γ , IL-2, IL-4, IL-13), Th migratory behaviour (CXCR3, CCR5, CCR4), Th signalling suppressors (SOCS1, SOCS3), transcription factor (T-Bet, GATA-3) and apoptosis (FasL/Fas, TNF/TNFR, XIAP, Bax, caspase-3) was analyzed by RT-PCR 6 h and 7 d post-irradiation. CD4⁺ and TUNEL positive cells were visualized by immunostaining.

RESULTS: The expression of Th1-related cytokine/chemokine receptors (IFN- γ , IL-2, CXCR3, CCR5) was repressed at 7 d post-irradiation while Th2 cell cytokine/chemokines (IL-4, IL-13, CCR4) were not repressed or even upregulated. The irradiation-induced Th2 profile was confirmed by the upregulation of both Th2-specific transcription factor GATA-3 and SOCS3. Although an apoptosis event occurred 6 h after 10 Gy of intestinal γ -irradiation, apoptotic mediator analysis showed a tendency to apoptotic resistance 7 d post-irradiation. CAPE amplified apoptotic events at 6h and normalized Bax/FasL expressions at 7 d.

CONCLUSION: CAPE prevented the ileal Th2 immune response by modulating the irradiation-influenced cytokine environment and apoptosis.

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INTRODUCTION

Radiation-induced acute enteritis is the major dose-limiting complication in the radiotherapy for cancers in the abdominal region^[1]. Irradiation injuries involve cytokine pathways^[2] that trigger an inflammatory process causing clinical symptoms (diarrhea, dismotility). The immune-mediated pathologic development is the consequence of sustained local cytokine production. In particular, the modification of the CD4⁺ helper T (Th) cell polarization induced by abdominal irradiation has not been investigated and these cells may play a primary role in irradiation injury. Crohn's disease and TNBS-induced colitis are both Th1-like forms of gut inflammation^[3] whereas infection with *Schistosoma* is associated with a Th2 immune response^[4].

Activated CD4⁺ Th cells have been divided into at least two major exclusive T cell subsets according to their cytokine profile. The cytokine environment has been proposed as the major variable influencing Th development^[5]. In the presence of IL-12, activated Th cells differentiate into Th1 cells, secreting predominantly IFN- γ and IL-2 which promote delayed-type hypersensitivity responses and pro-inflammatory responses. In contrast, the Th cells that are activated in the presence of IL-4 differentiate into Th2 cells, producing mainly IL-4 and IL-13 and promote humoral and allergic responses^[6]. In addition, IL-4 inhibits the expression of the β 2 subunit of the IL-12 receptor, thereby preventing a Th1-type response, whereas IFN- γ stimulates it^[7]. Thus, IL-12R β 2 expression is preferentially found in Th1 cells and maintains responsiveness to IL-12, whereas Th2 cell development is accompanied by a loss of this receptor subunit. Th1 and Th2 cells were originally distinguished from each other by their partially specific profile of cytokine expression. Analysis of such *in vivo* polarized Th cells has revealed differential sets of gene expression, including specific chemokine/chemokine recep-

tors. Typically, Th1 cells migrate to inflammatory sites in response to CXCR3 and CCR5 ligands^[8]. Likewise, CCR4 is preferentially expressed on Th2 cells. The restricted expression of cytokines by polarized Th cells arises from a differential expression of transcription factors. T-Bet, expressed specifically in Th1, mediates IFN- γ production and GATA-binding protein 3 (GATA-3) plays a pivotal role in Th2 phenotype development and in Th1 phenotype inhibition^[9]. However, recent studies have shown that cytokine signalling is under a negative feedback regulation by proteins called suppressors of cytokine signalling (SOCS). Thus, the differentiation toward the Th1 or Th2 pathway may be mediated in part by the selective repression of IL-12 or IL-4 signalling pathways^[10].

Inhibition of pro-inflammatory molecules provides a logical therapeutic option for patients with abnormal immune cell activation^[11]. This concept should be re-evaluated in light of evidence that immune activation is also regulated by apoptosis. Because programmed cell death controls the life span of cells, an apoptosis defect could result in an inappropriately long cell survival and a state of uncontrolled inflammation leading to chronic and autoimmune diseases^[12]. This may be the case in Crohn disease, where abnormal mucosal T cell reactivity is well documented^[11]. Medical or accidental exposure to radiation could lead to an impairment of specific immune responsiveness by apoptotic death of resting lymphocytes. The transcription factor NF- κ B is a key regulator of genes implicated in the immune/inflammatory response^[13]. Recently, we have shown that abdominal irradiation induced an inflammatory process involving NF- κ B activation^[3]. NF- κ B also regulates the expression of many genes involved in the control of cell proliferation and cell death *via* extrinsic factors [TNF- α , Fas Ligand (FasL), IL-2], specific receptors [TNF and Fas receptors (TNFR, Fas)] and intrinsic mediators (XIAP, Bax)^[14]. Targeting of inducible extrinsic factors has been proposed as a method for constraining immune responses by selectively killing activated T cells or inhibiting differentiation^[15].

The modulation of NF- κ B action by specific drugs is primarily based on cytokine synthesis. However, little is known about the effects of these drugs on Th1 or Th2-derived cytokine production patterns, on their specific transcription factors (T-Bet, GATA-3) and on chemokine receptors CXCR3/CCR5 (on Th1 cells) or CCR4 (on Th2 cells). Here, using abdominal γ -irradiation where both type-1 and type-2 cells can be activated, the pattern of gene expression was analysed in the ileum mucosa. To test the hypothesis that defective apoptosis is involved in inflammation process^[16] and orients the immune response following irradiation, we used the apoptotic and immune regulation properties of Caffeic Acid Phenethyl Ester (CAPE), an active phenolic compound inhibitor of NF- κ B^[17] to modulate the irradiation-influenced cytokine environment and the Th polarization at the dose previously used^[18]. This study was performed at 6 h post-irradiation where apoptotic events occurred, and at one week (d 7) corresponding to the restoration of the intestinal morphological structure.

MATERIALS AND METHODS

Animals and treatment

Male Wistar rats (Elevage Janvier, France), weighing 200-250 g, were housed with *ad libitum* food and water. All experiments were conducted in accordance with the French regulations for animal experimentation (Ministry of Agriculture Order No. 87-848, October 19, 1987). Anesthetized rats received a single abdominal dose of 10 Gy γ -irradiation (⁶⁰Co source). Control rats were sham-irradiated. Rats were divided into four groups ($n = 6$): controls, controls treated with CAPE, irradiated untreated, and irradiated CAPE treated. Rats were injected intraperitoneally with CAPE (30 mg/kg in saline containing 200 g/L Tween 80) 10 min before irradiation and once a day for 6 d^[18]. The animals were sacrificed and the ileal tissues were dissected at 6 h and 7 d after exposure. Entire ileum specimens were taken for immunostaining and only mucosa layers, separated from the muscularis by scraping, were kept at -80°C for protein extraction and RT-PCR.

Immunostaining

Ileal tissue samples were washed in PBS and then fixed in 40 g/L buffered formaldehyde solution. After dehydration, specimens were embedded in paraffin and sectioned (5 μ m).

CD4⁺ detection: Immunohistochemistry was performed to assess the presence of CD4 phenotype cells. Briefly, deparaffinized and rehydrated tissue sections were treated with 3 mL/L hydrogen peroxide in methanol to inhibit the endogenous peroxidase activity. Sections were incubated in protein blocking serum (Dako, USA) before treatment for 90 min at room temperature with the monoclonal anti-CD4 antibody (ab6413; 1/100 dilution, Abcam). Slides were then rinsed in 50 mmol/L Tris/HCl, 3 g/L NaCl and 1g/L Tween-20. The secondary reagent was the Vector Elite ABC kit (Dako).

Apoptotic cell detection: Apoptotic cells were visualized by the terminal deoxynucleotidyltransferase (TdT)-mediated dUTP-biotin nick-end labelling assay (TUNEL) using the *In Situ* Cell Death Detection kit (Roche Molecular Biochemicals, France) according to the manufacturer's instructions. Briefly, deparaffinized and rehydrated ileum tissue sections were incubated in proteinase K (20 mg/L in 10 mmol/L Tris-HCl, pH 7.6) for 10 min at 37°C. Endogenous peroxidase activity was quenched with blocking solution (3 g/L H₂O₂ in methanol). Sections were exposed to the TUNEL reaction mixture at 37°C for 1 h. After washing in PBS buffer, POD (peroxidase) was added and allowed to react for 30 min at 37°C. For both staining methods, the slides were treated with a NovaRED™ kit (Vector Laboratories Inc, Burlingame, CA) for colour development and counterstained with Meyer's hemalum.

Caspase-3 activity assay

Mucosal protein extracts were prepared by a Dounce homogenization protocol from 50 mg of tissue in a hypotonic buffer (25 mmol/L HEPES, pH 7.5, 5 mmol/L MgCl₂, 1 mmol/L EGTA, 1 mmol/L PMSF, 5 mg/L aprotinin and leupeptin). After centrifugation of the homogenates, the supernatants were collected. Five

Table 1 Sequences of custom primers for real-time quantitative PCR

Gene	Forward primer	Reverse primer
Th1 cytokines/receptors		
IFN- γ	CACGCCGCGTCTGGT	TCTAGGCTTTCAATGAGTGTGCC
IL-2	ATTTCCAGGCACCTGAAGATGTTT	CCCCATGATGCTCACGTTTA
IL-23	GCGTTCTCTTCCCGTTCCA	TGCTCCGTGGGCAAAGAC
IL-12R β 2	GGCTGCATCTCCATTACAGA	CTGCTTATTGGATGTGAGTTTTG
Transcription factors		
T-Bet	TCTGTCTCCAGCCGTTTCT	CGCTCACTGCTCGGAACTCT
GATA-3	GGCGGCGAGATGGTACTG	TCTGCCCATTCATTTATGGTAGA
Chemokine receptors		
CXCR3	AGGTCAGTGAACGTCAAGTGCTAG	CAAAAAGAGGAGGCTGTAGAGGA
CCR5	CGAACTTCTCCCAACAAA	CTTCTCTTCTGGACTCCCTACAACA
CCR4	GCCTCAAAGACAGACTTCCTTG	AGCGTTCGGTCTAGTTTCCAC
Apoptotic Extrinsic factor/receptors		
TNF- α	CATCTTCTCAAAATTCGAGTGACAA	TGGGAGTAGACAAGGTACAACCC
TNFR1	TGCCACGCAGGATCTTTCTAAG	TGGGGGTTGTGACATTTGC
TNFR2	CCCATCATTGAACCAAGCATCAC	AAGCAGGTCGCCAGTCTTAACATC
Fas	CGAATGCAAGGGACIGATAGC	TTCGAGCAGTGTGTGCGGTTT
FasL	AAGGCGGCCTGTGATCA	TTGCAAGACTGACCCCGG
CTLA-4	GTGGAACTCATGTACCCACCG	GCATGGTTCGGATCGATGAC
Apoptotic Intrinsic factors		
XIAP	GGTGCAAGAAGCTATACGAATGG	AGTTGCTCCCAGATGTTTGGAG
Bax	GCTGACCTTGGAGCAGCC	ATGTTGTGTCCAGTTCATCGC
Caspase-3	TGCGCCATGCTGAAACTG	CCTTCCGGTTAACACGAGTGA
Suppressors of Cytokine Signalling Proteins		
SOCS1	ACACTCACITCCGCACCTTC	AGCAGCTCGAAAAGGCAGTC
SOCS3	CTCCAGCATCTTGTGTC-GGAAGAC	TACTGGTCCAGGAACTCCCGAATG
Housekeeping		
HPRT	GCTCGAGATGTCATGAAGGAGA	TCAGCGCTTAATGTAATCCAGC

hundred μ g of extracted protein was incubated with 100 μ mol/L of the synthetic substrate Ac-DEVD-pNA in 500 μ l of 25 mmol/L HEPES (pH 7.5) containing 200 ml/L glycerol and protease inhibitors. After 20 min of reaction at 37°C, the caspase-3-like activity was assayed by measuring the absorbance at 405 nm.

Quantitative real-time RT-PCR

Real-time quantitative RT-PCR was used to measure the expression of Th1 (IL-23, IL-12R β 2, IFN- γ , IL-2, CXCR3, CCR5, T-Bet and SOCS1) or Th2-derived genes (IL-4, IL-13, CCR4, GATA-3, SOCS3), the cytokine TNF- α and its specific receptors (TNFR1, TNFR2), cytotoxic mediators (Fas/FasLigand, CTLA-4) and apoptotic genes (Bax, XIAP, caspase-3). Total RNA was prepared with the RNeasy total RNA isolation kit (Qiagen, France) according to the manufacturer's instructions. RNA integrity was confirmed by electrophoresis on a denaturing agarose gel and ethidium bromide staining. One μ g of total RNA was reverse transcribed with SuperScript II reverse transcriptase (Invitrogen, France) using random hexamers. IL-4 and IL-13 primers from the manufacturer were used to amplify first-strand cDNA with TaqMan technology (Applied Biosystems). PCR amplification of the other genes used SYBR PCR master Mix. The primer sequences (Table 1) have been designed by Primer Express software (Applied Biosystems). Optimized PCR used the ABI Prism 7000 Sequence detection system (Applied Biosystems). PCR fluorescent signals were normalized to the signal obtained from the housekeeping gene HPRT for each sample.

Immunoblot analysis

Total proteins were obtained by homogenization of ileal samples in a cold RIPA buffer containing classical protease-inhibitor cocktail. Total proteins (40 μ g) were boiled in SDS and mercaptoethanol buffer; then separated on a 120 g/L polyacrylamide gel (NuPAGE gels; Invitrogen, France) and electroblotted. Polyvinylidene difluoride membranes were incubated with a blocking solution (50 g/L skim milk in TPBS containing 1 mL/L Tween 20), washed with TPBS and incubated with rabbit polyclonal primary antibody directed against T-Bet for 1h at RT (dilution 1/200; Santa Cruz) or goat polyclonal GATA-3 overnight (dilution 1/100; Santa Cruz). After washing, immunodetection was performed with the respective horseradish-linked secondary antibody (1/2000; Amersham). Chemiluminescence was detected according to manufacturer's protocol (ECL, Amersham Biosciences).

Result expression and statistical analysis

We used the comparative $\Delta\Delta C_T$ -method^[19] for relative mRNA quantification of target genes, normalized to an endogenous reference (HPRT) and a relevant non-irradiated control, equal to $2^{-\Delta\Delta C_T}$. $\Delta\Delta C_T$ is the difference between the mean ΔC_T (irradiated sample) and mean ΔC_T (non-irradiated sample), where ΔC_T is the difference between the mean C_T (genes) and the mean C_T (HPRT). Each sample was monitored for fluorescent dyes, and signals were regarded as significant if the fluorescence intensity significantly exceeded (10-fold) the standard deviation of the baseline fluorescence, defined as threshold cycles (C_T).

All data are expressed as the mean \pm SD error of the

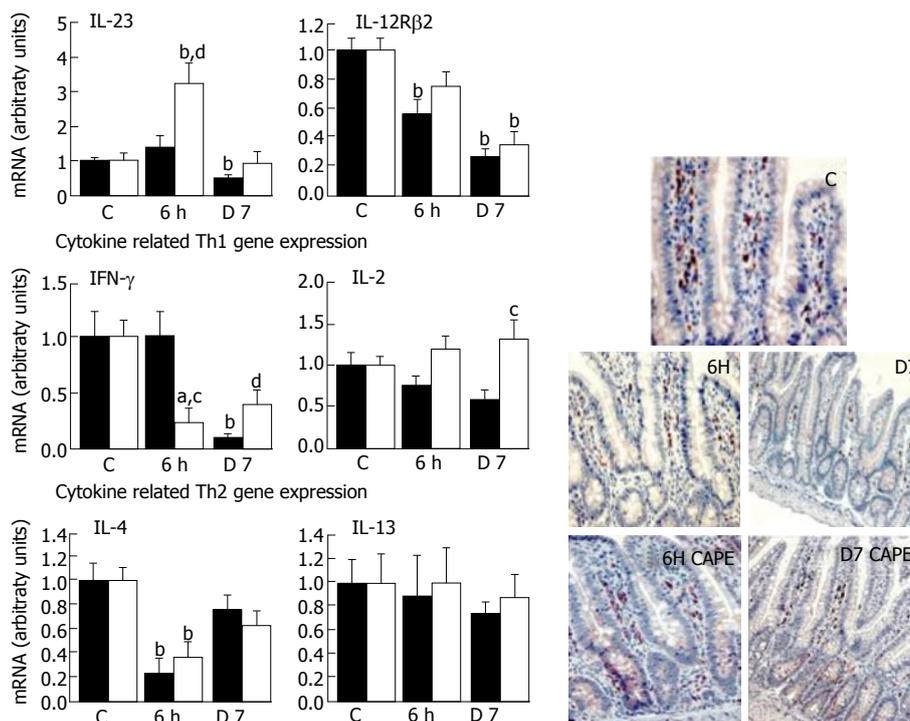


Figure 1 Th1/Th2-like pattern of gene expression in the ileal mucosal layer after γ -irradiation in rats. (■): CAPE-untreated, (□): CAPE-treated and CD4⁺ immunostaining in control (C), 6 h and 7 d (D7) after irradiation (D x 20) (mean \pm SEM, $n = 6$); ^a $P < 0.05$, ^b $P < 0.01$ vs control; ^c $P < 0.05$, ^d $P < 0.001$ vs untreated.

mean (SEM) for 6 animals. Comparisons between groups used Mann-Whitney test for non-paired data.

RESULTS

Effect of CAPE on the irradiation-modified mucosal cytokine environment

Activation and polarization of CD4⁺ Th cells was dependent on local dominant factors that control differentiation. IL-12 is a critical cytokine for polarizing naïve CD4⁺ T cells towards a Th1 dominance^[7]. Moreover, the T-cell responsiveness to this mediator depends on expression of the inducible IL-12Rβ2 subunit of the IL-12 receptor^[20]. IL-12Rβ2 is preferentially expressed by Th1 cells and is lost with Th2 cell development. From the analysis of IL-23 expression, the IL-12p40 form appears to be a key step in Th1 pathogenesis^[21], since irradiation repressed it ($P < 0.01$) and it fell to below the levels of IL-12Rβ2 at d 7 ($P < 0.01$) (Figure 1). Significant evidence exists for a relationship between IFN- γ expression and IL-23/IL-12R signalling since Th1 commitment is enhanced by IFN- γ ^[22]. Accordingly, the irradiation-induced down-regulation of IL-23/IL-12Rβ2 (-50% and -80%) was correlated with a drastically low level of IFN- γ expression (95% decrease, $P < 0.01$) at 7 d. These results suggest that irradiation did not induce the expression of a Th1 pattern of cytokines. In addition, the low IL-2 expression, the other Th1 cytokine representing one of the major growth factors for the expansion of activated T cells, observed 7 d after irradiation supports this hypothesis. Although CAPE did not significantly restore the radiation-induced decrease in IL-12Rβ2 expression, IFN- γ and IL-23 expression tended to return to normality and the IL-2 mRNA level was 1.5-fold increased as compared to controls.

To address the induction mechanism of Th2 dominance by irradiation, we examined the IL-4 and IL-13 mRNA

levels. As shown in Figure 1, irradiation only repressed IL-4 expression (-75%) soon after irradiation (6 h) but had no significant effect at 7 d on either IL-4 or IL-13 expression. CAPE did not influence IL-4 and IL-13 mRNA levels.

The presence of CD4⁺ T cells in the *lamina propria* was confirmed by immunostaining. The CD4⁺ T cell numbers were not modified at 6 h and 7 d post-irradiation compared to the control (Figure 1). CAPE did not influence the cell frequency.

Polarization of CD4⁺ T lymphocytes in ileal tissue following irradiation

To characterize the Th cell polarization, we exploited the fact that activated Th cells acquire and maintain at high levels a specific pattern of chemokine receptors: CXCR3/CCR5 present on Th1 or CCR4 on Th2. Irradiation modified the chemokine receptor profile. There was a 70% fall ($P < 0.01$) in CXCR3 levels at 6 h post-irradiation and a tendency for decline in CCR5, whereas CCR4 mRNA was about 2.5-fold ($P < 0.05$) up-regulated at both time-points after irradiation (Figure 2). CAPE significantly counter-balanced the CXCR3 mRNA repression ($P < 0.05$) and the CCR4 mRNA induction ($P < 0.01$) at d 7.

The transcription factor T-Bet is selectively expressed in Th1 cells whereas GATA-3 is selectively expressed in Th2 cells. Expression of these genes is rapidly induced in primary T cells triggering the Th1 or Th2 pathway^[23]. Our data showed that irradiation induced an early (6 h) significant decrease of T-Bet expression, followed by an accentuation of decline down to 20% of the control level at d 7 ($P < 0.005$). In contrast, GATA-3 was significantly up-regulated at 7 d ($P < 0.01$, Figure 3). Because T-Bet is reported to represent Th1 immune responses and GATA-3 those of Th2, it has been suggested that the ratio of expression of these transcription factor could

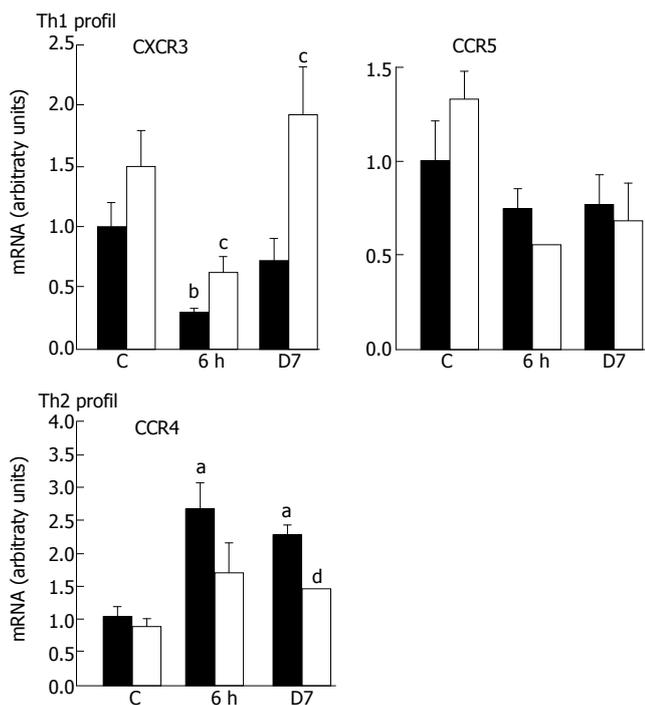


Figure 2 Expression of the chemokine receptors CXCR3/CCR5 and CCR4 post-irradiation in rats. (■): CAPE-untreated; (□): CAPE-treated (mean ± SEM, n = 6); ^aP < 0.05, ^bP < 0.01 vs control; ^cP < 0.05, ^dP < 0.01 vs untreated rats.

be used to reflect the Th1 or Th2 status in mixed cell populations^[24]. The increase in GATA-3 expression was greater than for T-Bet, which was reflected by the T-Bet/GATA-3 ratio with a 2-fold decrease at d 7 ($P < 0.01$). These data confirm that irradiation causes a shift toward Th2 polarization. CAPE significantly reduced ($P < 0.05$) the radiation-induced T-Bet repression and GATA-3 was totally down-regulated at d 7 normalizing the T-bet/GATA-3 ratio. Western blot analysis confirmed the increased T-Bet/GATA-3 ratio where the T-Bet protein level reduction detected 7 d post-irradiation was removed after CAPE treatment (Figure 3).

Polarization of CD4⁺ regulated by SOCS proteins

Endogenous feedback regulators of cytokine activities such as SOCS proteins regulate the amplitude and duration of the polarization influenced by T-Bet or GATA-3^[10]. Since Th1 cells predominantly express SOCS1 while Th2 express high levels of SOCS3, we have hypothesized that SOCS genes might contribute to the establishment of a stable Th1 or Th2 phenotype. The analysis of the SOCS expression revealed a drastic reduction of SOCS1 mRNAs at d 7, whereas SOCS3 mRNAs were significantly up-regulated at 6 h with a high level maintained (2-fold, $P < 0.05$) for one week (Figure 4). The functional consequences of a high level of SOCS3 expression and a relatively low abundance of SOCS1 may drive the majority of CD4⁺ toward a Th2 pattern of cytokine expression. CAPE limited the irradiation effect on SOCS1, with a restoration to half the level of that of the controls at 7 d ($P < 0.05$), but had no effect on SOCS3 expression.

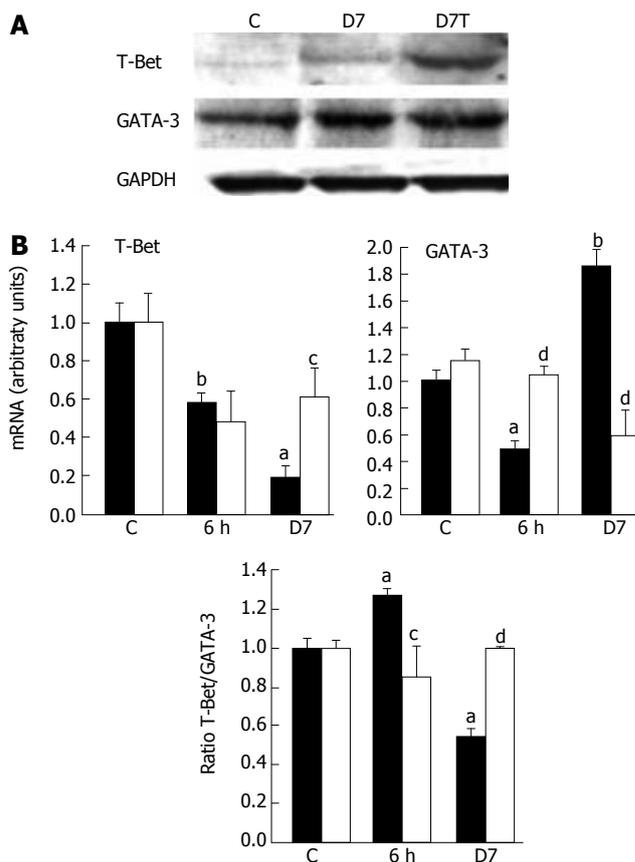


Figure 3 Expression of transcription factors of Th1/Th2 polarization after irradiation in rats. (■): CAPE-untreated; (□): CAPE-treated. A: Western blot analysis using T-Bet and GATA-3 polyclonal antibody, GAPDH levels are used as internal standard; B: Real-time RT-PCR analysis (mean ± SEM, n = 6); ^aP < 0.005, ^bP < 0.01 vs control; ^cP < 0.05, ^dP < 0.01 vs untreated rats.

Early administration of CAPE modified apoptotic events in irradiated rats

Since the decision as to whether T cells survive or undergo apoptosis is of particular importance for adaptive immune responses^[14], we examined the expression of genes associated with the regulation of apoptosis, in the mucosa after irradiation. Real-time PCR was used to quantify mRNA levels of extrinsic factors (TNF-α, FasL, CTLA-4), specific receptors (TNFRs, Fas) and intrinsic mediators (XIAP, Bax, caspase-3). Irradiation led to an over-expression of TNF-α (20-fold at 6 h, $P < 0.001$), which correlated with a significant increase in TNF receptor (R1, R2) mRNAs (Figure 5). These over-expressions remained significant at d 7. It has been demonstrated that CAPE has anti-inflammatory and apoptotic properties^[3,17]. Accordingly, CAPE significantly reduced TNF-α up-regulation at 7 d post-irradiation and led to a dual effect on TNFR2 expression with an early (6 h) amplification of expression and, in contrast, a reduction of the irradiation effect at 7 d.

The engagement of the death receptor Fas by FasL plays an important role in apoptotic events. Our data have shown that irradiation has a biphasic effect with a significant increase of Fas and FasL mRNA levels at 6 h and a significant FasL repression at d 7 ($P < 0.05$). CAPE over-expressed Fas/FasL at 6 h with a persistent effect

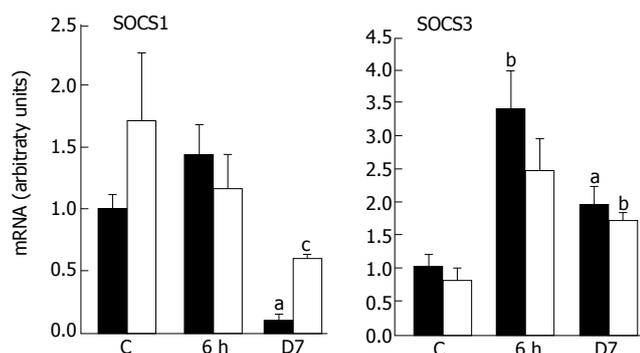


Figure 4 Expression of the regulator cytokine gene SOCS1 and SOCS3 after irradiation in rats. (■): CAPE-untreated; (□): CAPE-treated (mean \pm SEM, $n = 6$); ^a $P < 0.05$, ^b $P < 0.01$ vs control; ^c $P < 0.05$, ^d $P < 0.01$ vs untreated.

on FasL mRNA level at d 7 (Figure 5). In association with Fas/FasL, CTLA-4 engagement could potentiate the elimination of pathogenic T cells^[24]. Irradiation significantly down-regulated CTLA-4 at d 7 (90% decrease compared to controls, $P < 0.01$), which was not modified by CAPE (Figure 5). These results suggest that an absence of cytotoxic mediators occurs at 7 d post-irradiation that is minimized by CAPE-induced FasL normalization.

The modulation of the members of the IAP (apoptosis inhibitors) family, such as the anti-apoptotic protein XIAP or the pro-apoptotic protein Bax, regulates apoptosis. No significant change in XIAP mRNA was observed at 6 h whereas Bax expression rose at 6 h then progressively decreased at 7 d leading to a 70% reduction ($P < 0.01$) (Figure 6). The apoptotic event was confirmed at 6 h by an increase in caspase-3 activity that contrasted with the normal activity found at 7 d; caspase-3 expression remained invariant. CAPE reduced the XIAP expression and over-expressed Bax as compared to irradiated rats at 7 d ($P < 0.01$), resulting in a significant caspase-3 activity increase ($P < 0.01$).

The irradiation-induced apoptosis events at 6 h were confirmed by TUNEL assay (Figure 6A-E). TUNEL⁺ cells were sparse in the lamina propria of controls, and were only located at the top of the villi. Six hours post-irradiation, the number of apoptotic cells was considerably increased and localized in the crypts and the bottom of the villi compared to controls. Some apoptotic cells appeared in the lamina propria. The irradiation-induced apoptotic effect was amplified in the crypts and the bottom by CAPE at 6 h but not at d 7, as compared to controls.

DISCUSSION

In this study, we investigated whether targeting defective mucosal cell death underlies the sustained therapeutic benefit of CAPE by orienting the immune response induced by irradiation. The results show firstly that irradiation led to an earlier apoptotic process, which progressively resulted in an apoptotic resistance and secondly, that irradiation oriented the immune response to a predominance of Th2 type polarization. Overall, the emerging picture is that, in the non-inflamed gut, the T cells manifest increased spontaneous apoptosis mediated by the Fas pathway that

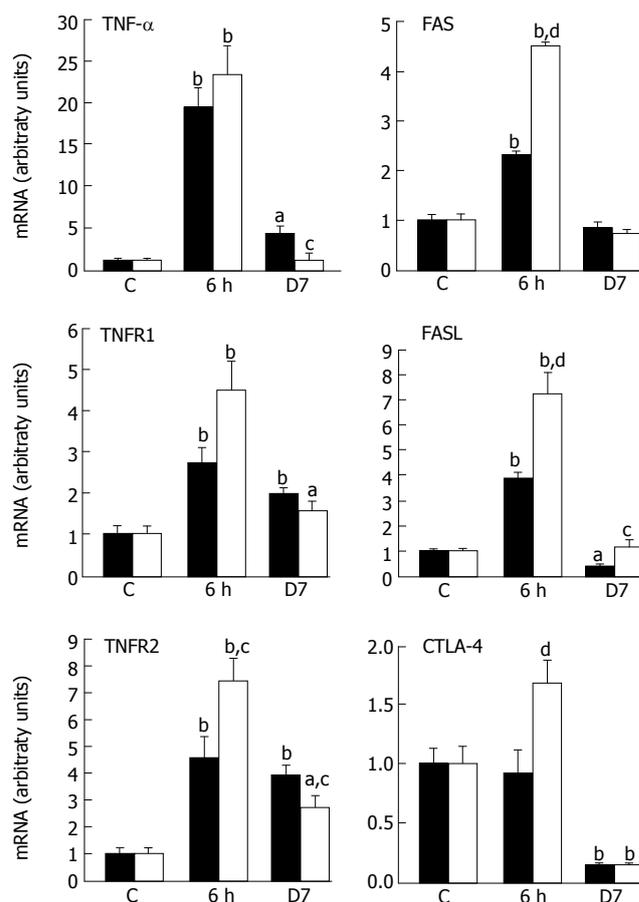


Figure 5 Irradiation effect on extrinsic apoptotic factors TNF- α , FasL and related-receptors expression post-irradiation in rats. (■): CAPE-untreated; (□): CAPE-treated (mean \pm SEM, $n = 6$); ^a $P < 0.05$, ^b $P < 0.01$ vs control; ^c $P < 0.05$, ^d $P < 0.01$ vs untreated rats.

limited expansion of T cells. By contrast, in the inflamed tissue, resident T cells are relatively resistant to apoptosis. Thus, they exhibit an enhanced cytokine production because of prolonged survival, which significantly aggravates the inflammation. The functional importance of T cell apoptosis resistance has been emphasized by recent studies in animal models of IBD showing that IL-12 and TNF- α antibodies appear to suppress chronic intestinal inflammation by the induction of T cell apoptosis^[25]. The regulation of T cell activation by immunosuppressive drugs is known to be relevant in the treatment of inflammatory disorders. Medical or accidental exposure to radiation leads to an inflammatory process^[2]. Currently, the purpose of using NF- κ B inhibitors as an adjuvant for cancer treatment is to increase the therapeutic index of radiotherapy and chemotherapy^[26,27]. The success of this approach relies both on its ability to promote cancerous cell killing by irradiation and to spare normal cells from enhanced damage. By analysing the immunomodulatory and apoptotic properties^[17], we have shown that CAPE shifts from the radiation-induced Th2-like pattern of gene expression toward Th1 gene expression by an apoptotic sensitization.

Irradiation led to the repression of genes involved in 4 key features of Th1 cell differentiation: genes involved in Th1 differentiation (IL-12, IL-12R β 2), genes associated with Th1 cytokine responses (IFN- γ , IL-2), genes

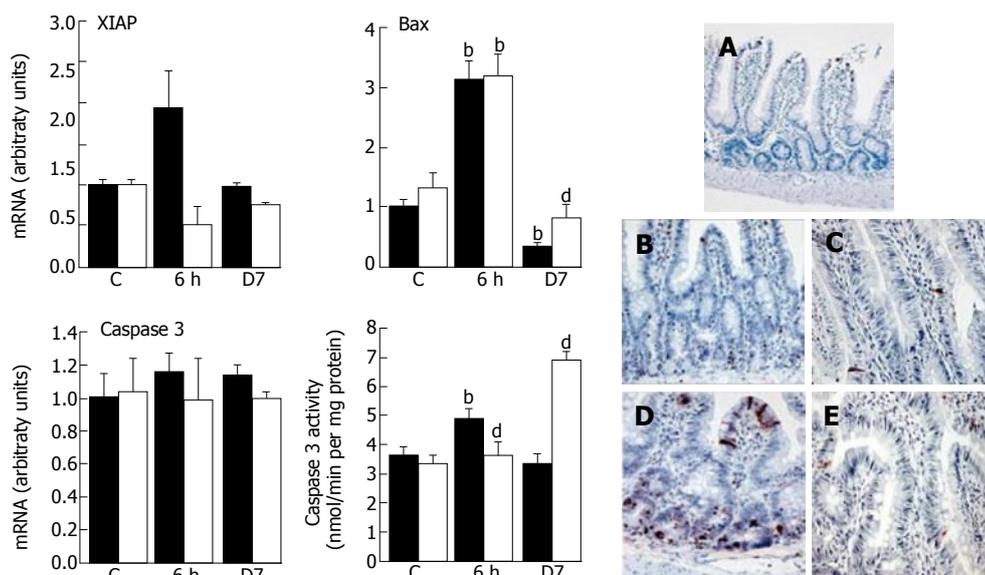


Figure 6 Irradiation effect on intrinsic apoptotic factor expression modulation and caspase activity post-irradiation in rats. (■): CAPE-untreated (□): CAPE-treated. Apoptotic cells presence was confirmed by the terminal deoxynucleo tidyltransferase (TdT)-mediated dUTP-biotin nick-end labeling (TUNEL) staining in ileum. (A): Control; (B): 6 h; (C): 7 d after γ -irradiation. (D): 6 h; (E): 7 d from irradiated CAPE-treated (x 20) (mean \pm SEM, $n = 6$); ^b $P < 0.01$ vs control; ^d $P < 0.01$ vs untreated.

regulating cell death and apoptosis (Fas/FasL, CTLA-4) and genes controlling Th1 migratory behaviour (CXCR3, CCR5). Simultaneous to the down-regulation of Th1-associated chemokine receptors, the transcription factor T-Bet was moderately expressed while GATA-3 was up-regulated. Transcription factors such as T-Bet and GATA-3 play a critical role in the initiation of primary immune responses and skewing the response toward the Th1 or Th2 pathway^[23]. The duration of the response depends on the coordination of lymphocyte responses. These aspects of the adaptive immune response are regulated by endogenous feedback regulators of cytokine activities^[10]. Thus, SOCS proteins are differentially expressed: Th1 cells predominantly express SOCS1 whereas Th2 cells express very high levels of SOCS3. The restricted pattern of expression in Th1 and Th2 cells underscores the importance of SOCS members in Th cell subtypes. A preferential up-regulation of SOCS1 expression in Th1 but not Th2 after IL-12 stimulation argues for a role of cytokine signalling in SOCS regulation^[10,22]. In our study, we found a differential expression of SOCS at 7 d post-irradiation with a repression of SOCS1 and an overexpression of SOCS3. The functional consequence of SOCS3 expression may be to drive the majority of CD4⁺ toward a Th2 pattern of cytokine expression at this time. However, cytokines can override the effects of SOCS3 during Th differentiation. In fact, a major effect of IL-12 signalling in non-polarized Th cells is to down-regulate SOCS3 expression in cells committed to the Th1 lineage. In this way, Th1 differentiation requires IL-12/IL-12R β 2 signalling^[28] and SOCS1^[24], and the drastic repression of IL-23, closely related to IL-12, that occurred at 7 d post-irradiation reduces the possibility of a Th1 lineage. In addition, IL-12/IL-12R β 2 signalling supports high IFN- γ production and IFN signalling to down-regulate IL-4 and prevent Th2 dominance^[29]. In this way, although IL-4 expression induced by irradiation was not elevated, the protection of Th1 dominance after irradiation could not be maintained by the mere fact of the IFN- γ repression at d 7. Szabo *et al*^[7] have shown that when IFN- γ production is limited, a low IL-4 production in early responses may critically inhibit IL-12R β 2 expres-

sion with a significant effect on the development of a Th1/Th2 shift. In fact, only low IL-4 level is required in the complete absence of extrinsic Th1 signals (e.g; IL-12) to generate Th2 CD4⁺. These results showed the same prevalence of an irradiation-induced Th2 response in the lung. In fact, enhanced lymphocyte reactivity, dominated by Th2 cells, has been shown in radiation-induced pneumonitis and subsequent pulmonary fibrosis, suggesting a critical role for Th2 CD4⁺^[30].

TUNEL staining has revealed an increase of apoptotic cells observed at the top of the villi and in the crypts. Some apoptotic cells appeared after irradiation in the lamina propria which is formed from a majority of immune cells. In fact, a differential sensitivity of lymphocyte subpopulations to irradiation-induced apoptosis has been reported; in particular, CD4⁺ T cells are relatively radioresistant^[31]. In this work, CD4⁺ immunostaining confirmed their presence after irradiation. TUNEL positive cells increased at 6 h confirming the radiosensitization effect of CAPE but without real modification of the number of CD4⁺ cells. In this study and in accordance with other findings^[27], the earliest intestinal response to radiation injury reflects a complex series of apoptotic events involving extrinsic factors (TNF- α , FasL, IL-2), specific receptors (TNFR, Fas) and intrinsic mediators (XIAP, Bax and caspase-3). All these apoptotic events occurred rapidly after irradiation (6 h) but were amply repressed one week post-irradiation.

The extrinsic factors such as Fas and FasL are particularly important in T cell apoptotic events. The observed decrease in Fas/FasL expression may mediate T cells apoptosis and becomes especially important in the irradiation-inflammatory process at d 7, because failures of this mortality pathway can impair both autocrine and paracrine mechanisms for eliminating activated T cells. In fact, Fas is expressed by the both Th cell subsets whereas only Th1 cells express detectable amounts of FasL^[29]. The differential expression of FasL by Th1 and Th2 cells is correlated with the ability of these cells to undergo to apoptosis: Th1 clones are highly sensitive to apoptosis, whereas Th2 clones are relatively resistant. In this way, the genesis of Th2 orientation may occur early (6 h) post-

irradiation. In view of the ability of CAPE to induce an apoptotic event *via* Fas activation^[32], the restoration of FasL expression by CAPE may suggest a modification of the apoptosis resistance and of the Th1/Th2 balance at 7 d post-irradiation. This hypothesis must be confirmed by *in vitro* experiments. Interestingly in respect to asthma, it has been shown that intratracheal delivery of adenovirus encoding FasL could significantly suppress pulmonary Th2 immune response^[33].

Fas, a member of the TNF receptor family, is inducible by TNF- α itself^[34], a good correlation was observed concerning the radiation-induced expression variation between Fas/FasL and TNF- α . CAPE did not alter this high mRNA level at 6 h, but this correlation disappeared at 7 d with a repression of TNF- α and a normalization of FasL levels. The cell death signal induced by TNF- α is mediated by TNFR1. In contrast, the engagement of TNFR2 promotes cell survival by the induction of anti-apoptotic molecules^[35]. Although TNF- α was repressed at 7 d, the TNFR1 up-regulation induced by irradiation was not altered by CAPE in contrast to TNFR2 which was down-regulated. This observation, associated with FasL normalization, may be an indication of a limitation of apoptosis resistance induced by CAPE at 7 d. Intrinsic gene product analysis confirmed this argument at this time when CAPE also normalized Bax expression, contributing to increased caspase-3 activity.

Cell death is a complex series of events regulated by the intrinsic propensity of individual cells, the environment and cell surface. The regulation of T-cell activation/apoptosis depends on critical cell surface molecules, in particular CTLA-4 which down-regulates T cell activation. The immune importance of CTLA-4 has been reported *in vivo* where CTLA-4 blockade exacerbates some autoimmune diseases^[36] and CTLA-4-deficient mice exhibit a massive polyclonal increase in T cells. On the basis of these properties and in view of our data which showed that CTLA-4 was totally repressed at 7 d, it seems possible that an irradiation-induced T-cell activation effect may have occurred. CAPE had no effect on CTLA-4 expression, so that it seems probable that the T cell expansion/activation was maintained, but an orientation of the immune response cannot be excluded.

These results suggest that CAPE, by modulating the cytokine pattern, changed the orientation of immune responses after irradiation and favoured a Th1 response notably by the total or partial suppression of IL-12 and IFN- γ repression. Interestingly, CAPE could inhibit T-cell activation by targeting both NF- κ B and the nuclear factor of activated T cells (NFAT)^[37]. Diehl *et al.*^[38] showed that NFAT activation in CD4⁺ is required for the development of Th2 differentiation and its inhibition in T cells prevents the Th2 immune response in a model of allergic pulmonary inflammation. The activation of NFAT by irradiation still has not been demonstrated but we cannot exclude its potential involvement in the irradiation-induced Th2-like profile.

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Expression and significance of ICAM-1 and its counter receptors LFA-1 and Mac-1 in experimental acute pancreatitis of rats

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development of AP from a local disease to a systemic illness. Upregulation of ICAM-1, LFA-1, Mac-1 and subsequent leukocyte infiltration appear to be significant events of pancreatic and pulmonary injuries in AP.

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Key words: Acute pancreatitis; ICAM-1; LFA-1; Mac-1

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Abstract

AIM: To investigate the role of intercellular adhesion molecule-1 (ICAM-1) and its counter receptors LFA-1 and Mac-1 in acute pancreatitis (AP).

METHODS: SD rats were allocated to AP group and control group randomly (25 rats each). AP was induced by infusion of 5% chenodeoxycholic acid into the pancreatic duct, followed by ligation of pancreatic duct. The rats were sacrificed at 1, 3, 6, 12 and 24 h after induction of pancreatitis. Five rats were sacrificed at one time point in the two groups before the blood and specimens from pancreas and lung were obtained. Serum amylase and ascitic fluid were measured at each time point. Expression of ICAM-1 at different time points was assessed by immunohistochemistry in pancreas and lung, and the expression of LFA-1 and Mac-1 on neutrophils at different time points was detected by flow cytometer.

RESULTS: Induction of AP was confirmed by the serum levels of amylase and histological studies. The expression of ICAM-1 in pancreas increased significantly than that in the control group at all time points ($P < 0.05$ or $P < 0.01$), as well as the expression in lung except at 1 h. The expression of LFA-1 and Mac-1 on neutrophil in blood increased significantly in AP group than that in control group at several time points ($P < 0.05$ or $P < 0.01$). The amount of ascitic fluid and serum amylase level of AP group increased significantly than that of control group at all time points ($P < 0.05$ or $P < 0.01$). Parallel to these results, a significant neutrophil infiltration was found in pancreas and lung tissues of AP group rats.

CONCLUSION: Our findings suggest the important role for ICAM-1, LFA-1 and Mac-1 in mediating the

INTRODUCTION

Acute pancreatitis (AP) is a disease of variable severity. Approximately 80% of patients have a relatively mild attack that resolves with few or no complications. The mortality rate is low. However, 20% of patients in whom pancreatic necrosis develops may incur systemic complications with a mortality rate as high as 40%^[1]. Recent investigations have established that one of the earliest pathophysiologic events in pancreatitis is the colocalization of acinar cell organelles containing digestive and lysosomal enzymes, resulting in premature intracellular activation of proteases^[2,3]. The individual pancreatic cell injury becomes magnified and propagated by inducing impaired microcirculation, leukocyte adhesion, and leukocyte infiltration. These become central events in the pathogenesis of pancreatic necrosis and extrapancreatic complications^[4,5]. Organ dysfunction occurs in one of four patients with acute pancreatitis, and 60% of them die from pulmonary damage^[6].

Intercellular adhesion molecule-1 (ICAM-1) is expressed on endothelial cells and is responsive to numerous inflammatory mediators^[7]. It mediates both leukocyte adhesion and migration through the endothelium into tissues^[8]. Lymphocyte function-associated antigen 1 (LFA-1) and Mac-1 are the counter receptors of ICAM-1, both of which are expressed on leukocytes and are related with the above mediating activities of ICAM-1^[9].

The aim of the present study was to analyze the mechanism of upregulation and levels of expression of

ICAM-1 in the pancreas and lung tissues of AP, to detect the expression of LFA-1 and Mac-1 on neutrophils, and to explore the role of these adhesion molecules in the development of AP from a local disease to a systemic illness.

MATERIALS AND METHODS

Animals, anesthesia and surgical procedures

Sprague-Dawley rats (250 to 300 g) were fasted 12 h before the experiment but were allowed free access to water. Fluothane, oxygen and nitrous oxide were used in surgical anesthesia (2 L/min). All rats underwent a 2-cm-long midline laparotomy, and the pancreatic duct was visualized entering the duodenum. The duct was cannulated and AP was induced by retrograde intraductal injection of 5% chenodeoxycholic bile acid (0.1 mL/kg, Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 2 min. After injection, the pancreatic duct was ligated and the abdomen was closed.

Experimental design

A total of 50 rats were allocated to control group and AP group (25 rats in each). The rats of control group only received laparotomy without retrograde intraductal injection of 5% chenodeoxycholic bile acid of pancreas and the pancreatic duct ligation. Blood samples (2 mL) were drawn through inferior vena cava. Pancreatic and lung tissues were removed and processed as indicated below before the rats were killed at 1, 3, 6, 12 and 24 h after induction of pancreatitis ($n = 5$ per group). Ascitic fluid was quantified by estimation of weight of towels after absorption of the ascitic fluid from the abdominal cavity (assuming 1 g = 1 mL).

Amylase in serum

One milliliter blood was sampled to measure amylase in plasma using the RaBA-SUPER (Kyoto First Science Ltd., Kyoto, Japan) kit.

Tissue immunohistochemical staining

For immunohistochemical staining, pancreatic and lung tissues were immediately snap-frozen in Freon precooled in liquid nitrogen and embedded in OCT embedding medium (Tissue-Tek, Miles Inc, Elkhart, USA). Cryostat sections (5 μ m) were fixed in acetone for 10 min, and endogenous peroxidase activity was quenched by incubation of methanol with 0.3% H₂O₂ for 5 min. After rinsing with 0.02 mol PBS, the sections were incubated with primary anti-ICAM-1 antibody (Santa Cruz Biotechnology, Inc., California, USA) diluted at 1:400 in antibody dilution (Dako Corporation, Carpinteria, CA, USA) for 10 min in moist chamber at room temperature. Unbound antibodies were washed from the tissue three times in PBS for 3 min, incubated for 10 min with the secondary biotinylated anti-mouse IgG antibody (LSAB II kit, Dako Corporation, Carpinteria, CA, USA), and then washed three times for 3 min. The sections were incubated with the LSAB reagent (LSAB[®] 2 System, HRP KIT, DAKO Corporation, Carpinteria, CA, USA) for 10 min, washed and developed

in DAB solution. Finally, the slides were fixed in alcohol, counter-stained 3 times with hematoxylin for 10 min, and covered with DAKO Glycergel. NIH Image 1.61/ppc software was used to detect the density of positive area of slides.

Flow cytometry analyses

Blood (1 mL) was anticoagulated with 2% EDTA (0.2 mL). Leukocyte-rich plasma was obtained by centrifugation for 10 min at 1500 r/min. The erythrocytes were then lysed 15 min with 4 mL of hemolysin twice. The leukocyte pellet was washed once with PBS and resuspended with 1 mL of PBS. Leukocytes suspensions (100 μ L) were mixed with 20 μ L of FITC-labeled monoclonal antibody anti-LFA-1 (CD11a/CD18) (BD Pharmingen, San Diego, CA, USA), FITC-labeled anti-Mac-1 (CD11b/CD18) (BD Pharmingen, San Diego, CA, USA), and FITC-labeled IgG (Sigama, Saint Louis, Missouri, USA) that acted as a nonspecific control antibody. It was then incubated at 4°C for 30 min, washed twice in ice-cold PBS, resuspended in 1 mL of PBS and kept on ice until analyzed. Flow cytometry was performed on an FACScan (Coulter Epics XL, USA). Data were collected for forward scatter, side scatter, and fluorescence on the FL1 channel (530 nm) with Coulter Elite software. Cells were analyzed with a gate setting for neutrophils and twenty thousand cell counts were accumulated for analysis. Green fluorescence (FL1) was determined and mean fluorescence intensity (MFI) was calculated. The nonspecific binding (IgG) in control group and AP group was measured at MFI values of 2.81 (0.57) and 3.74 (0.97) units, respectively.

Statistical analysis

Results are expressed as means and standard errors of the mean. Differences between AP and control groups were judged by using the Student's *t* test for parametric data. *P* values of less than 0.05 were considered significant.

RESULTS

Serum amylase

In AP group rats, there was a marked increase in serum amylase from 1 to 24 h with a peak value after 6 h (Figure 1).

Ascitic fluid

The amount of ascitic fluid in AP group increased significantly than that in control group at the same time point (Figure 2).

Histological findings

A low level of ICAM-1 expression was observed in the pancreas and lung tissues in control rats. ICAM-1 expression in the pancreas of AP rats was significantly increased above control levels at 1 h, and continued to increase until 12 h and remained elevated at that level for the whole observation period (24 h) (Figure 3).

ICAM-1 expression in the lung of AP rats was significantly increased above control levels at 3 h, and continued to increase until 24 h (Figure 4). Immunohistochemical analysis showed that overexpression of ICAM-1 was localized in small venules and capillaries.

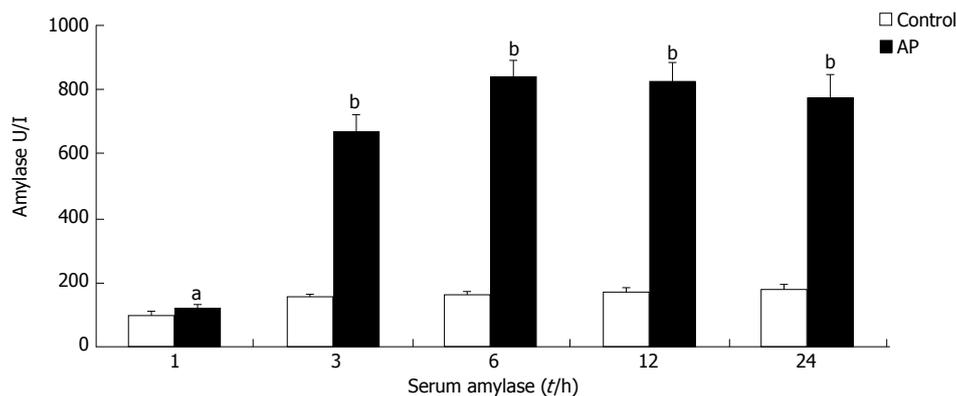


Figure 1 The time course of serum amylase. ^a $P < 0.05$ vs control at the same point, ^b $P < 0.01$ vs control at the same point.

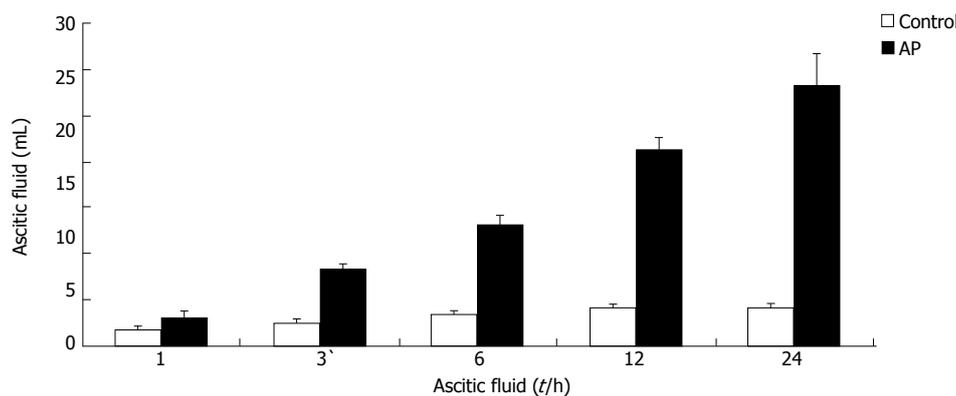


Figure 2 The time course of ascitic fluid output. ^b $P < 0.01$ vs control at the same time point.

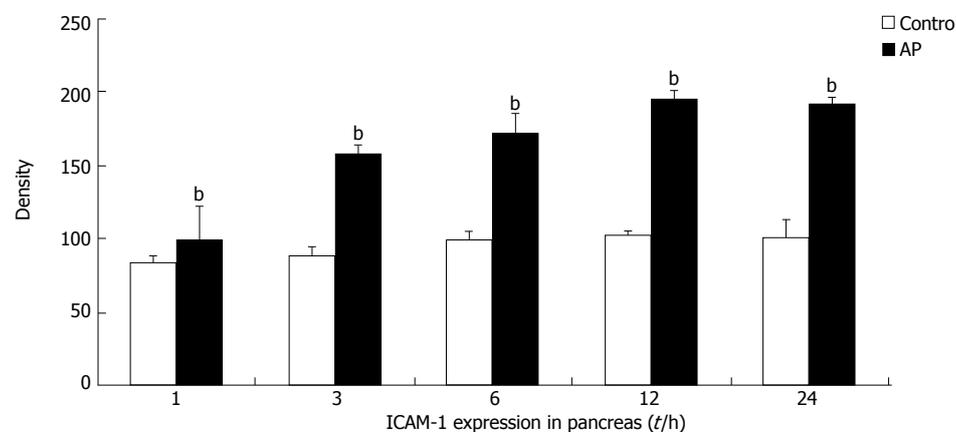


Figure 3 ICAM-1 expression in pancreas. ^b $P < 0.01$ vs control at the same time point.

Expression of LFA-1 and Mac-1 on leukocytes

The expression of LFA-1 and Mac-1 on leukocytes in AP group increased significantly than that in the control group (Figure 5).

DISCUSSION

Intra-acinar cell activation of digestive enzyme zymogens is generally believed to be an early event in pancreatitis. Most researchers, however, believe that subsequent nonacinar cell phenomena, including the activation and recruitment of inflammatory cells, play a critical role in determining the ultimate severity of the disease. Nonacinar cell phenomena including the local generation and release of proinflammatory factors and activation of circulating inflammatory cells are also believed to couple pancreatic

injury to distant organ complications, such as lung injury.

Leukocyte sequestration within areas of injury and inflammation is a multistep process that begins with leukocyte activation. It involves the adhesion of circulating activated inflammatory cells to microvascular endothelial surfaces, and culminates in the transmigration of those cells across the endothelial barrier and into the involved tissue. Currently available evidence indicates that the adhesion of activated leukocytes, including neutrophils, to endothelial surfaces results from the interaction of leukocyte surface proteins, such as CD11/CD18, with endothelial cell surface adhesion molecules such as ICAM-1.

Surprisingly, little attention has been paid to probing the relationship between the ICAM-1 and its counter receptors (LFA-1, Mac-1). Furthermore, the time course of LFA-1

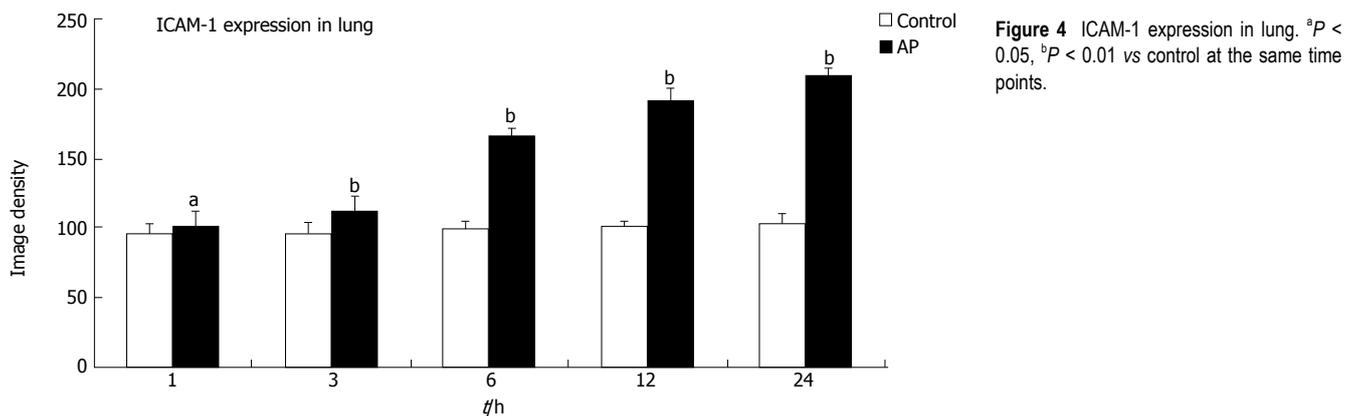


Figure 4 ICAM-1 expression in lung. ^a*P* < 0.05, ^b*P* < 0.01 vs control at the same time points.

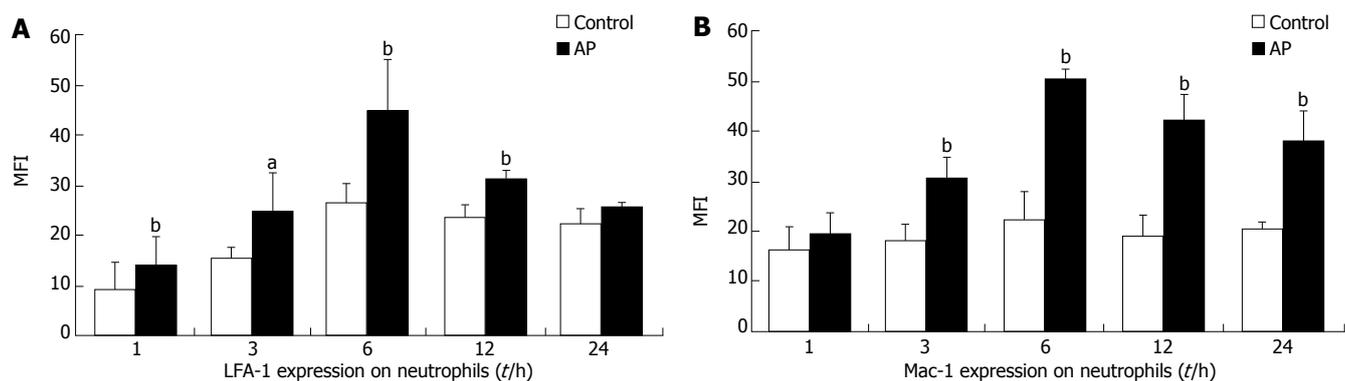


Figure 5 LFA-1 (A), Mac-1 (B) expression on neutrophils. ^a*P* < 0.05, ^b*P* < 0.01 vs control at the same time point.

and Mac-1 expression in severe acute pancreatitis has not been studied. We serially investigated LFA-1 and Mac-1 expression on circulatory neutrophils and ICAM-1 expression in pancreas and lung tissues of AP rats in the current study.

ICAM-1 is an adhesion molecule expressed on endothelial cells that mediates both firm adhesion of leukocytes to the endothelium and transmigration and infiltration of marginated leukocytes into the organs^[7]. Endothelial ICAM-1 is enhanced by hypoxia^[10] and cytokines, including, among others, interleukin-6, interleukin-8 and tumor necrosis factor- α ^[2,7]. These inflammatory mediators, oxygen free radicals, and cytokines respond early in the pathogenesis process of acute pancreatitis^[2,11,12].

ICAM-1 is a counter receptor for LFA-1(CD11a/CD18) and Mac-1(CD11b/CD18). The interaction between ICAM-1 and CD11/CD18 is a major determinant of leukocyte adhesion to endothelial cells^[13], a phenomenon that is itself a prelude to leukocyte transmigration across the endothelial barrier and into areas of inflammation.

The multistep model of leukocyte emigration provides a conceptual framework describing the molecular recognition mechanism that leads from cell capture to adhesion strengthening and transmigration. Neutrophils use selectins to tether to inflamed endothelium and subsequently arrest at septic sites in the vasculature through binding of the β_2 -integrins^[14,15]. The β_2 -integrins family members LFA-1 and Mac-1 are involved in firm adhesion of neutrophils to endothelial cells^[16]. Intravital

microscopy in mice and rats has shown that CD11a and CD11b each contribute significantly to firm adhesion, and concurrent blocking of both β_2 -integrins family is required for complete inhibition of human and canine neutrophil transmigration^[17-19]. Furthermore, the adhesion complex CD11b/CD18, induced by inflammatory cytokines, has been demonstrated to play a crucial role in neutrophil-induced tissue damage by enhancing neutrophil migration and adhesiveness^[20].

We probed the expression of adhesion molecules such as ICAM-1 and its counter receptors, LFA-1 and Mac-1 in AP. We found expression of ICAM-1 was markedly upregulated in AP, as well as the expression of LFA-1 and Mac-1 on neutrophils. ICAM-1 is also upregulated in organs beyond the pancreas in severe pancreatitis. Our experiments showed an increased expression of ICAM-1 in the endothelium of venules and capillaries within the lungs at 6 h in AP, followed by massive leukocyte infiltration. The results of this study imply the important roles for ICAM-1 and its counter receptors LFA-1 and Mac-1 in the pathogenesis of pancreatic and systemic injury in acute necrotizing pancreatitis. The current studies are consistent with the concept that expression of ICAM-1, LFA-1 and Mac-1 in the pancreas during AP is a critical link in the development of tissue injury and organ dysfunction. Although overexpression of inflammatory mediators do play an important role in pathogenesis but the molecular mechanisms of the whole cascade, i.e. cell surface molecules, signaling into the cell and resulting response of the cell has still not been worked out. It

may be possible to prevent tissue injury and necrosis and thereby to minimize the possibility for subsequent secondary infection by administering antibodies or other drugs in AP. Further studies are in progress to define the window of opportunity.

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CLINICAL RESEARCH

Comparison between empirical prokinetics, Helicobacter test-and-treat and empirical endoscopy in primary-care patients presenting with dyspepsia: A one-year study

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cost-effective option.

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Abstract

AIM: To investigate the optimal strategy to treat dyspeptic patients in primary care.

METHODS: Dyspeptic patients presenting to primary care outpatient clinics were randomly assigned to: (1) empirical endoscopy, (2) *H pylori* test-and-treat, and (3) empirical prokinetic treatment with cisapride. Early endoscopy was arranged if patients remained symptomatic after 2 wk. Symptom severity, quality-of-life (SF-36) as well as patient preference and satisfaction were assessed. All patients underwent endoscopy by wk 6. Patients were followed up for one year.

RESULTS: Two hundred and thirty four patients were recruited (163 female, mean age 49). 46% were *H pylori* positive. 26% of *H pylori* tested and 25% of empirical prokinetic patients showed no improvement at wk 2 follow-up and needed early endoscopy. 15% of patients receiving empirical cisapride responded well to treatment but peptic ulcer was the final diagnosis. Symptom resolution and quality-of-life were similar among the groups. Costs for the three strategies were HK\$4343, \$1771 and \$1750 per patient. 66% of the patients preferred to have early endoscopy.

CONCLUSION: The three strategies are equally effective. Empirical prokinetic treatment was the least expensive but peptic ulcers may be missed with this treatment. The *H pylori* test-and-treat was the most

INTRODUCTION

Dyspepsia, defined as pain or discomfort centered in the upper abdomen, is a common complaint, affecting 18.5% of the population in our locality^[1]. Underlying pathology ranges from functional dyspepsia, peptic ulcer to gastrointestinal cancer. Among the various aetiologies, functional dyspepsia is the most common diagnosis in community-based patients^[2]; Up to 79% of dyspeptic patients referred from primary care for open-access endoscopy have normal endoscopic findings^[3].

Primary care medicine is the first point of presentation for dyspeptic patients. Studies have suggested that dyspepsia alone accounts for 20%-70% of all gastrointestinal consultations with general practitioners; up to one-third of these patients may eventually be referred to a gastroenterologist^[4]. The financial burden for diagnosis and treatment of this common condition is therefore great.

It is unclear how dyspepsia should be investigated and treated in primary care settings, but there are several common approaches. Empirical endoscopy would theoretically be the gold standard in diagnostic accuracy, but it is expensive and invasive. Empirical H₂ receptor antagonist therapy has been extensively studied, but has not been shown to have superior cost-benefit over empirical endoscopy^[5]. In addition, there may be concerns that antisecretory treatment may mask symptoms of

gastric cancer and lead to a delay in diagnosis.

Another possible approach is to test for *H pylori*. The close association between *H pylori* and chronic gastritis that accompanies peptic ulcer disease and gastric cancer offers an opportunity to screen for organic disease based on the presence of the bacterium.

An alternate approach is empirical prokinetic treatment. Studies have demonstrated that cisapride and domperidone are beneficial in the treatment of functional dyspepsia over placebo^[6-8]. By adopting this approach, the majority of patients with functional dyspepsia could be treated appropriately whereas the smaller number of patients with organic dyspepsia may not benefit from this treatment. Persistence of dyspepsia in this group would then lead to endoscopy and diagnosis. The small delay in diagnosis is not unreasonable since, in real life, many primary care doctors would give antacids to dyspeptic patients without alarm features and monitor symptoms on follow-up. Cisapride has been withdrawn from most markets since this study was carried out though related drugs such as mosapride are still in use in some countries.

In this study, we investigated empirical endoscopy, *H pylori* test-and-treat, and empirical prokinetic therapy as possible diagnostic and treatment strategies in primary-care patients presenting with dyspepsia.

MATERIALS AND METHODS

Patients aged 18 or older presenting with a primary complaint of previously uninvestigated dyspepsia in four government-run primary-care clinics in Hong Kong were recruited for the study. Dyspepsia was defined as pain or discomfort centered in the upper abdomen^[9]. History and physical examination were carried out by the primary care physician.

Exclusion criteria for this study were current intake of drugs other than antacids for dyspepsia, previous peptic ulcer disease, history of gastric surgery, presence of malignancy within the previous five years, history of intake of aspirin or non-steroidal anti-inflammatory drugs in the previous four weeks, presence of alarm symptoms, such as weight loss and gastrointestinal haemorrhage, and clinical suspicion of an organic cause of dyspepsia. In addition, those with a family history of sudden death, history of palpitations, co-existing intake of drugs that may interact with cisapride and those with an abnormal electrocardiogram were excluded.

After obtaining written consent for the trial, a locally-validated 13-C urea breath test was administered in the primary care clinic. The details of the breath test have been described elsewhere, and had a sensitivity and specificity of 96.5% and 97.7% respectively^[10]. The results were not disclosed to the patients until after the trial unless the patients were randomised into the *H pylori* test-and-treat group.

The patients were subsequently reassessed by one of the investigators in the gastroenterological unit of Queen Mary Hospital within 24 h of initial presentation. Inclusion and exclusion criteria were checked and a complete physical examination was performed. Electrocardiography was also performed on all recruited patients. A previously

validated Chinese (Hong Kong) version of the SF-36 health survey^[11], as well as a validated twelve-item dyspepsia symptom severity score^[12] were administered. Patients were then randomised using a computer-generated sequence into one of three investigation and treatment groups as detailed below.

This study was approved by the research and ethics committee, faculty of medicine, the University of Hong Kong.

Empirical endoscopy

Patients in this group received endoscopy on the initial day attending the gastrointestinal unit. Oesophagogastroduodenoscopy was performed by one of the investigators and an endoscopic diagnosis was made. A rapid urease test was performed if the patient had peptic ulcer disease and biopsies for histology were taken for gastric ulcers and suspicious lesions.

Patients with organic diagnoses were treated accordingly. *H pylori* eradication therapy was given to those with peptic ulcers or erosive gastritis with a positive test for the bacterium.

Dyspeptic patients with normal endoscopy or non-erosive gastritis were considered to have functional dyspepsia and were treated with cisapride 5 mg three times daily for six weeks.

Helicobacter test-and-treat

Patients who tested positive for *H pylori* in the 13C urease breath test received omeprazole 20 mg twice daily, amoxicillin 1 g twice daily, and clarithromycin 500 mg twice daily. The local eradication rate for this regimen is 87.8%^[13]. Patients who tested negative for *H pylori* were treated as functional dyspepsia and given cisapride 5 mg three times daily for six weeks.

Empirical prokinetics

All patients in this group were given cisapride 5 mg three times daily for six weeks regardless of *H pylori* positivity (Figure 1).

Follow-up

Patients were followed-up at two weeks, six weeks and one year after the initial treatment. At follow-up the dyspepsia symptom score and SF-36 survey were administered by an assessor blinded to the purpose of the study. Satisfaction of patients in regard to treatment was assessed at wk 6 using a 4-point Likert scale ranging from dissatisfied to very satisfied. Patients were asked whether they preferred endoscopy or a trial of drug treatment as the initial approach to their dyspepsia.

If the symptoms had not improved after two weeks of treatment, endoscopy was performed on group 2 and 3 patients. Organic pathology discovered at endoscopy was treated appropriately. For patients who had empirical endoscopy, no further investigation was offered unless there was a change in symptoms that raised suspicion of organic disease. At the end of the study, all patients who had not previously received endoscopy received an oesophago-duodenogastroscopy for a definitive diagnosis.

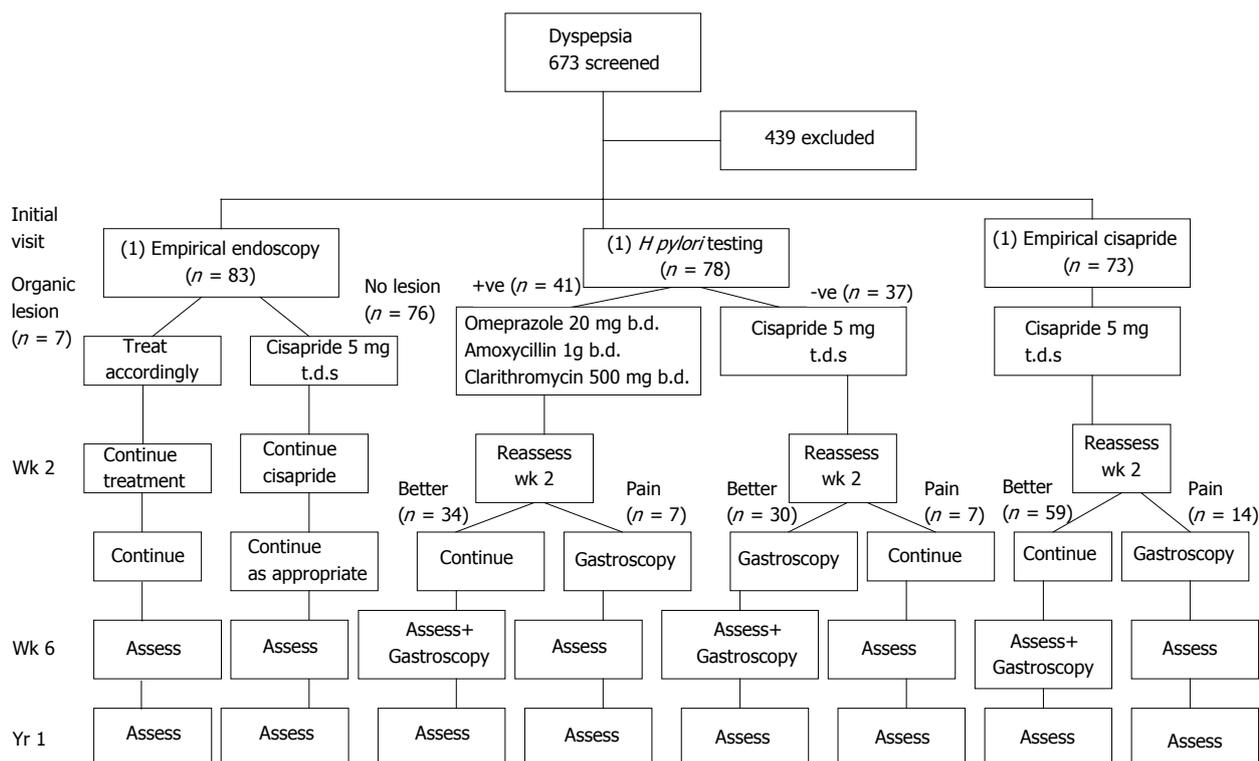


Figure 1 Flow chart for the three diagnostic and treatment arms.

Sample size

Sample size was determined using NCSS PASS 2002 (NCSS statistical software, Kaysville, Utah, USA). A previous study designed to develop dyspepsia severity scores found a mean of 20.7 and standard deviation of 3.8 in a local population of dyspeptic patients. The difference in mean symptom score between patients who felt better and those who did not was 1.2^[12]. Based on this result we estimated that 70 patients in each of the three groups would report a two-point difference in the mean symptom score between one group and the other two with a power of 0.9 and significance level of 0.05.

Cost analysis

Cost analysis was performed to examine the economic implications of the three strategies studied. The analysis was carried out from the provider's perspective, given all three treatment regimens were ambulatory procedures and therefore, assumed to incur similar direct personal, indirect and intangible costs to the patients and society generally. Costs were calculated according to the gazetted list of fees and charges of the Hong Kong Hospital Authority and hospital formulary costs.

Statistical analysis

The study was analysed according to intention-to-treat. Comparison of baseline dyspepsia scores and quality-of-life domains was made using one-way ANOVA and the Kruskal-Wallis test. Correlation between baseline dyspepsia scores and quality-of-life domains was calculated using Kendall's tau. Quality-of-life and dyspepsia symptom scores after treatment were compared among the three groups using a repeated measures procedure. The null

hypotheses regarding the main effects of time points and three treatment groups, and the interaction between treatment groups and different time points were tested using multivariate analysis. Age and sex were also included as covariables. Statistical analyses were performed using SPSS version 11.0 for windows (SPSS inc., Chicago, Illinois, USA).

RESULTS

Patients and demographic characteristics

Six hundred and seventy three patients were screened for inclusion in the study, of which 234 were recruited (mean age 49, range 18-79, 163 female). Reasons for exclusion included dyspepsia under treatment (257), refusal to participate (49), previous investigations for dyspepsia (51), non-steroidal antiinflammatory agent intake^[25], previous gastric surgery or peptic ulcer history^[34], alarm symptoms^[12], abnormal electrocardiogram^[6] and other malignancy^[3]. Baseline characteristics of the three groups of patients are as tabulated in Table 1. All patients completed the six-week treatment assessment period. One hundred and eighty one (77.4% overall; 77.1%, 69.2% and 86.3% in the three respective groups) attended one-year follow-up.

Diagnoses

Group one patients had endoscopy before commencing treatment and provided the most accurate data of the underlying diagnoses of dyspepsia. Among the 83 patients receiving empirical endoscopy 76 (91.5%) had normal findings or non-erosive gastritis, two (2.4%) had gastric erosions, four (4.8%) had peptic ulcer and one (1.2%) had

Table 1 Baseline characteristics

Group	Mean age	Sex (M/F)	<i>H pylori</i> positive (%)	Mean baseline dyspepsia score
Empirical endoscopy (n = 83)	52.5 ± 13.2	25/58	37 (44.6%)	20.4 ± 4.0
<i>H pylori</i> test-and-treat (n = 78)	46.5 ± 14.2	24/54	41 (52.6%)	20.8 ± 3.8
Empirical prokinetics (n = 73)	48.6 ± 13.0	22/51	30 (41.1%)	19.6 ± 3.8
P value	0.01 ^a	0.99	0.35	0.16

^aP < 0.05.

oesophagitis. Endoscopic findings for the three groups are summarised in Table 2. Overall, 46% of patients were *H pylori* positive.

Fourteen patients (18%) randomised to the *H pylori* test-and-treat group had persistent dyspepsia after two weeks of treatment and needed early endoscopy. None of these patients had organic dyspepsia; two had non-erosive gastritis and 12 had normal endoscopy. At six weeks, three patients with symptom resolution after treatment were found to have duodenal ulcers and three had gastroduodenal erosions. All of these patients were originally tested to be positive for *H pylori* and had received appropriate eradication therapy, but the ulcer had not completely healed by the time of endoscopy.

Fourteen patients (19%) randomised to empirical prokinetic therapy had persistent dyspepsia at two weeks follow-up and received early endoscopy. Eleven of these patients had normal endoscopy results or non-erosive gastritis, two had gastroduodenal erosions and one had a gastric ulcer. More importantly, at the final diagnostic endoscopy, two patients were found to have erosions, duodenal ulcer and gastric ulcer among those who reported symptomatic improvement after treatment. If endoscopy was not performed, these organic lesions would have likely been missed.

All gastric ulcers detected in this study were benign.

Symptom severity

The mean baseline dyspepsia severity score was 20.3 ± 3.9 and was not significantly different among the three groups studied (Table 1). After six weeks of treatment, there was a decrease in mean symptom score to 15.0 ± 2.9 (5.6, 5.6 and 4.4 for empirical endoscopy, *H pylori* testing and empirical cisapride, respectively). On one year follow-up, the mean dyspepsia score increased to 19.7 ± 7.2. Repeated measures analysis showed no significant effect of treatment group on dyspepsia severity. However, patients with organic causes of dyspepsia were less symptomatic than those with non-ulcer dyspepsia after one year (Table 3).

Quality-of-life

The SF-36 health-related quality-of-life scores for the various domains are tabulated in Table 4. At baseline,

Table 2 Endoscopic findings in the three groups n (%)

	Normal	Gastritis/duodenitis	Gastric erosions	Gastric ulcer	Duodenal ulcer	Oesophagitis
Empirical endoscopy (n = 83)	49 (59)	27 (32.5)	2 (2.4)	3 (3.6)	1 (1.2)	1 (1.2)
<i>H pylori</i> test-and-treat (n = 78)	61 (78.2)	10 (12.8)	4 (5.1)	0	3 (3.9)	0
Empirical prokinetics (n = 73)	46 (63.0)	17 (23.3)	4 (5.5)	3 (4.1)	3 (4.1)	0

Table 3 Mean dyspepsia symptom score by initial diagnosis

	Baseline	Wk 6	Yr 1
Non-ulcer dyspepsia (n = 210) ¹	20.3	15.0	20.1
Duodenal Ulcer (n = 7)	19.1	13.0	13.7
Gastric ulcer (n = 6)	23.3	18.7	14.4
Oesophagitis (n = 1)	17.0	12.0	17.0
Gastric erosion (n = 10)	29.0	15.0	12.0
All causes organic dyspepsia (n = 24) ¹	20.5	15.1	16.3

¹P = 0.045 between the two groups with age and sex as covariables.

there was no difference in quality-of-life between the three groups of patients. There was a significant correlation between the severity of dyspepsia and the BP, GH, MH, RE, RP, SF and VT domains, but not the PF domain at baseline. At one-year follow-up, there was a general decline in quality-of-life when compared to 6 wk follow-up. Repeated measures analysis again showed no significant effect of treatment group on quality of life.

Cost analysis

The calculated six-week investigation and treatment costs for empirical endoscopy, *H pylori* test-and-treat and empirical prokinetic therapies are HK\$4343, \$1771 and \$1750 per patient, respectively.

Patient satisfaction and preference

Overall, 81% of patients were either satisfied or very satisfied with treatment. There was no significant difference in satisfaction among the three diagnostic paradigms that were tested (Table 5). 66% of patients preferred to have endoscopy before taking medication; the differences in scores were again not significant among groups (Table 5).

Side effects of cisapride

No patient given cisapride reported significant palpitations or other side effects. All patients were able to complete the six-week course of treatment.

DISCUSSION

Underlying diagnoses of dyspepsia range from peptic ulcer, gastric cancer to functional dyspepsia, and it may

Table 4 Median SF-36 quality-of-life scores for the three diagnostic and treatment groups (wk 0/ wk 6/ one year). Transformed scores out of 100

	BP	GH	MH	PF	RE	RP	SF	VT
All groups	63/84/51	40/47/42	72/72/60	95/95/85	100/100/67	50/100/75	88/88/75	45/50/50
Empirical endoscopy	62/84/51	50/52/50	72/72/60	95/95/85	100/100/67	75/100/75	75/100/75	50/45/50
<i>H pylori</i> test-and-treat	62/84/51	40/45/36	68/72/60	95/95/80	67/100/33	50/100/75	75/88/63	45/45/45
Empirical Prokinetics	72/84/52	39/49/45	76/72/64	95/95/85	100/100/67	50/100/75	88/88/75	45/50/50

BP: bodily pain; GH: general health; MH: mental health; PF: physical functioning; RE: role-emotional; RP: role-physical; SF: social functioning; VT: vitality.

be difficult to determine cause without investigation. Age, symptom assessment and even the presence of alarm symptoms appear to be poor predictors of underlying pathology^[14]. However, despite the prevalence and potential high economic cost of this condition, the optimal strategy to manage dyspepsia in primary care practice remains unclear^[15].

Community-based studies have generally indicated that peptic ulcer and cancer are uncommon causes of dyspepsia^[2], accounting for only 20% of patients referred to open-access endoscopy^[3]. Therefore, it is reasonable that any investigative and treatment strategy should be effective in functional dyspepsia, representing the bulk of patients. The optimal approach to investigation and treatment would aim to: (1) provide the most efficacious and cost-effective treatment for functional dyspepsia, (2) allow for prompt treatment of patients with peptic ulcer, with *H pylori* eradication, if appropriate, (3) correctly diagnose malignant lesions so that definitive treatment can be given and (4) to avoid unnecessary treatment for patients with functional dyspepsia.

Several treatments have generally been shown to be better than placebo in the treatment of functional dyspepsia. Meta-analysis studies have estimated a 20% gain of H2 antagonist treatment against placebo^[16], but there may not be global improvement in symptoms^[17]. Proton pump inhibitors may benefit dyspeptic patients with co-existing reflux^[18]; a study reported 10% therapeutic gain over placebo treatment but efficacy seems to be limited to patients with ulcer-like symptoms^[19]. Cisapride has also been extensively evaluated in studies, though many studies had methodological deficiencies^[20]. Meta-analyses have generally found a therapeutic gain of 36%-39% when compared to placebo^[16,21] but publication bias may have contributed to these results. *H pylori* eradication is likewise controversial, with some studies showing a long-term benefit^[22], and others showing no benefit^[23].

Various options have been suggested in the investigation and treatment of patients presenting with dyspepsia. The American Gastroenterological Association has suggested that patients over the age of 45 years or those with alarm features should have empirical endoscopy^[24]. For younger patients without alarm symptoms, there may be several options: (1) empirical medical therapy (antisecretory drugs or prokinetics) with investigation for therapeutic failures, (2) immediate diagnosis with endoscopy, (3) testing for *H pylori* and studying positive subjects, and (4) testing for *H pylori* and

Table 5 Patient satisfaction and preference among the three diagnostic and treatment groups *n* (%)

	Very satisfied	Satisfied	Neutral	Dissatisfied	Prefer initial endoscopy
Empirical endoscopy (<i>n</i> = 83)	7 (8.4)	59 (71)	14 (17)	3 (3.6)	63 (75.9)
<i>H pylori</i> test-and-treat (<i>n</i> = 78)	6 (7.7)	53 (67.9)	14 (17.9)	5 (6.4)	48 (61.5)
Empirical prokinetics (<i>n</i> = 73)	6 (8.2)	56 (76.7)	8 (11.0)	3 (4.1)	43 (59.0)

treating positive cases with eradication^[24]. An Asian Pacific guideline also suggested a trial of antisecretory drugs or prokinetics for 2-4 wk^[25]. Among the alternatives, empirical prokinetic treatment has not been evaluated in previous studies.

Bytzer randomised 414 patients into groups receiving empirical endoscopy or ranitidine^[5]. Empirical treatment was associated with higher costs of ulcer drug use and a greater number of sick days. It was also notable that 33% of patients had organic causes of dyspepsia, compared to 8% in the present study. Another study comparing *H pylori* test-and-treat with endoscopy found greater patient satisfaction with endoscopy but comparable symptoms and sick leave days between the two groups^[26]. Comparative costs depend on individual centres. An American study reported similar costs between *H pylori* testing and empirical endoscopy^[27] whereas another claimed lower costs for *H pylori* eradication^[28]. A decision analysis supported a trial of proton pump inhibitor before endoscopy or test-and-treat^[29]. Test and endoscopy in primary care has been shown to increase endoscopy rates without benefit in symptom relief or quality of life^[30]. It is of note, however, that proton pump inhibitors may not be as efficacious in Asian populations; a paper using the same outcome instruments as the present study failed to show any benefit of four-week lansoprazole treatment over placebo in patients with functional dyspepsia^[31].

Most of the studies comparing different diagnostic strategies concentrated on cost. Less data is available concerning symptom resolution and quality of life. Many dyspeptic patients presenting for medical care have a fear of serious disease and malignancy^[32]. In addition

they are more anxious than non-presenters with similar complaints^[1]. It is possible that a completely normal endoscopy may relieve some of the anxiety. Indeed a study on barium meal in dyspeptic patients who were referred from general practice showed that the examination increased management confidence and allayed patients' anxiety^[33]. In this study, however, we failed to show any significant difference in symptom relief among the three groups, indicating that the comforting effect of a negative endoscopy may not be an important factor in the cure of functional dyspepsia.

We have calculated the cost of treatment and empirical endoscopy is most costly, followed by *H pylori* test-and-treat and the lowest cost was for empirical prokinetics. The high cost for empirical endoscopy in the present study is related to the relatively low rates of endoscopy for patients randomised to the other groups. In the *H pylori* test-and-treat and empirical cisapride groups, 18% and 19% of patients respectively had persistent dyspepsia after two weeks of treatment and needed early endoscopy. In Bytzer's study, 136 out of 206 patients randomised to empirical H₂ blocker therapy had therapeutic failure and required endoscopy^[5]. The difference may be due to differences in length of follow-up and also the lower rate of organic pathology in our study. In our population with dyspepsia, 8.5% had organic causes (empirical endoscopy group), compared with 33% prevalence of organic dyspepsia reported by Bytzer. The overall rate of organic dyspepsia is also lower than the rate in our previous study on open-access endoscopy, where 21% had organic pathology^[5]. The difference probably reflects differences in referral patterns, with family physicians referring more severe cases of dyspepsia for immediate endoscopy in the previous study.

We observed a high relapse rate of dyspepsia generally, with the mean dyspepsia severity score returning to the initial level on one-year follow-up. This is due to non-ulcer/functional dyspepsia being the predominant diagnosis in 92% of patients. Functional dyspepsia is a chronic disease that persists in patients; a study reported that 74% of dyspeptic patients are still symptomatic two years after the initial diagnosis^[34]. In contrast, the few patients with peptic ulcer in this study tended to remain asymptomatic after the ulcer has healed and these patients in fact have a better prognosis as far as symptom relapse is concerned.

Both empirical endoscopy and *H pylori* testing appeared to be safe options with similar patient satisfaction. All patients with organic dyspepsia were tested to be positive for *H pylori* and had appropriate eradication therapy. However, the main drawback in the study was the absence of malignancy; it was not possible to predict whether gastric cancers would have been missed by any approach. Although rare in younger patients, gastric cancers may present without sinister symptoms. A Canadian study reported a prevalence of 1.05 per thousand patients under 45 years presenting with dyspepsia without alarm symptoms^[35]. We may expect higher numbers in Asia, with a higher population prevalence of gastric cancer. The possibility of missing or delay in diagnosis of gastric malignancies needs to be considered in empirical treatment

approaches.

Since all patients with duodenal or gastric ulcer were positive for *H pylori*, another approach may be performing endoscopy only on those with a positive 13C urea breath test. Such an approach may prevent 54% of endoscopies. However, 60% of the patients with gastric erosions were negative for *H pylori*; inappropriate treatment of these patients may potentially lead to progression into ulcer disease.

There is limited data on empirical prokinetic treatment. Because of association with cardiac arrhythmia, the drug has been withdrawn in most markets. However, related drugs, such as mosapride, are still used in some countries and this study may throw light on the effectiveness of other prokinetic agents. In the present study we found this strategy to be associated with the lowest cost but with similar outcome regarding symptomatic improvement. However, interestingly, although prokinetic treatment was not targeted for the treatment of peptic ulcers, six patients (2 gastric ulcer, 2 duodenal ulcer, 2 gastric erosions) reported symptomatic improvement after cisapride alone and would not have received definitive therapy. Because of the dangers of misdiagnosing patients with peptic ulcers, empirical cisapride therapy could not be recommended. Despite concerns over potential QT interval prolongation and ventricular arrhythmia, a relatively low dose of 5 mg three times daily was not associated with any adverse event in our sample of patients.

In conclusion, most patients presenting to primary care physicians with dyspepsia appear to have non-ulcer dyspepsia, which is associated with a high one-year relapse rate. Empirical endoscopy, *H pylori* test-and-treat and empirical prokinetic treatments appear to have similar efficacy in improvement of dyspepsia and quality-of-life. Empirical prokinetics may result in organic lesions being missed in diagnosis. Most patients preferred having endoscopy first, if given the choice, but this is the most expensive option. *H pylori* test-and-treat is the most cost-effective safe treatment.

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Effects of chronic therapy with non-steroidal anti-inflammatory drugs on gastric permeability of sucrose: A study on 71 patients with rheumatoid arthritis

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well tolerated in chronic NSAID users.

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Key words: Non-steroidal anti-inflammatory drugs; Gastric mucosal permeability; Gastrointestinal toxicity; Sucrose test; Pantoprazole

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Abstract

AIM: To evaluate the gastric permeability after both acute and chronic use of non-steroidal anti-inflammatory drugs (NSAIDs) and to assess the clinical usefulness of sucrose test in detecting and following NSAIDs-induced gastric damage mainly in asymptomatic patients and the efficacy of a single pantoprazole dose in chronic users.

METHODS: Seventy-one consecutive patients on chronic therapy with NSAIDs were enrolled in the study and divided into groups A and B (group A receiving 40 mg pantoprazole daily, group B only receiving NSAIDs). Sucrose test was performed at baseline and after 2, 4 and 12 wk, respectively. The symptoms in the upper gastrointestinal tract were recorded.

RESULTS: The patients treated with pantoprazole had sucrose excretion under the limit during the entire follow-up period. The patients without gastroprotection had sucrose excretion above the limit after 2 wk, with an increasing trend in the following weeks ($P = 0.000$). A number of patients in this group revealed a significantly altered gastric permeability although they were asymptomatic during the follow-up period.

CONCLUSION: Sucrose test can be proposed as a valid tool for the clinical evaluation of NSAIDs-induced gastric damage in both acute and chronic therapy. This technique helps to identify patients with clinically silent gastric damages. Pantoprazole (40 mg daily) is effective and

INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) are largely consumed among the general populations worldwide, representing one of the most prescribed drugs^[1-6]. The main factor limiting their use is a high rate of adverse events, especially gastrointestinal toxicity^[7]. Although only a small number of patients develop serious gastrointestinal complications, they represent a large number of patients due to the widespread use of NSAIDs^[7-9].

Gastrointestinal toxicity is often clinically silent even in high risk groups due to the intrinsic analgesic activity of NSAIDs^[10]. Therefore aspecific symptoms such as dyspepsia, heartburn, nausea, vomiting or abdominal pain are frequently ignored by patients suffering, for example, from a chronic pain due to the underlying osteoarticular diseases.

A recent meta-analysis indicates that the association between use of NSAIDs and dyspepsia is unclear because of the variability of the terminology used in literature to report GI symptoms^[11]. Taking the "strict" definition of dyspepsia into account, based solely on epigastric pain-related symptoms, NSAIDs increase the risk of dyspepsia of 36%, which is useful for creating standard definitions. Endoscopy for diagnosis of NSAIDs-induced upper gastrointestinal damage is precise, sensitive and easy to perform, but it cannot be proposed as a screening test,

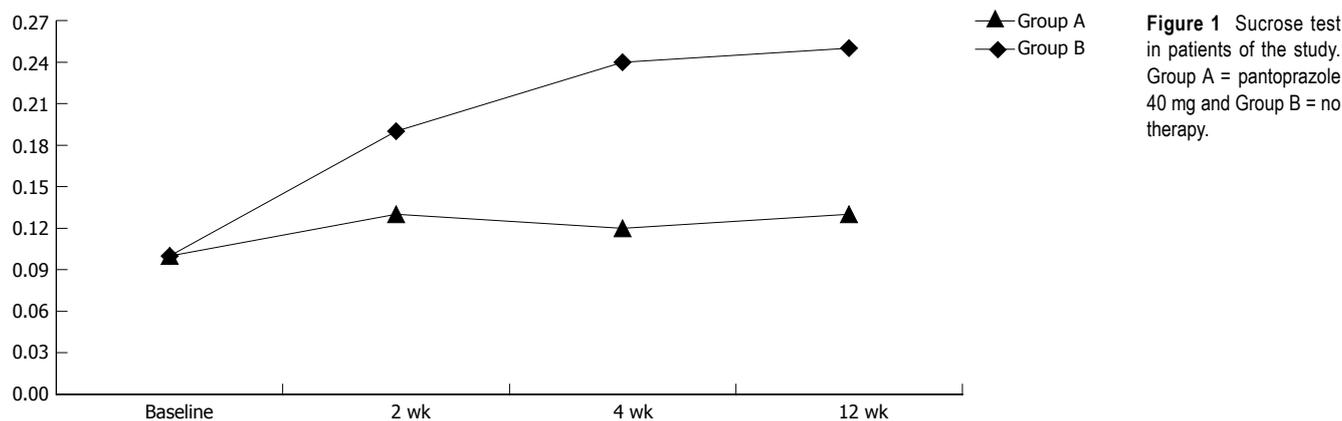


Figure 1 Sucrose test in patients of the study. Group A = pantoprazole 40 mg and Group B = no therapy.

especially in asymptomatic patients. Gastrointestinal permeability is usually tested to assess the small bowel function in many diseases such as coeliac disease and inflammatory bowel disease^[12,13]. Sucrose test is a simple, economic and non-invasive test for the assessment of gastric permeability and correlates with alterations detected with histology as previously demonstrated^[14]. Some authors have proposed sucrose test as an index for monitoring hypertensive gastropathy, confirming its clinical usefulness. Which is the best strategy in preventing NSAIDs-related adverse events in the gastrointestinal tract is still controversial^[16].

Many clinical trials have analyzed the efficacy and safety of concurrent administration of NSAIDs and misoprostol, H₂ blockers or proton pump inhibitors (PPIs) in reducing the occurrence of serious gastrointestinal events.

Available data show that PPIs are well tolerated and effective in preventing and treating non-steroidal anti-inflammatory drug-related mucosal lesions of the gastrointestinal tract^[17-20].

The aim of this study was to establish the clinical usefulness of sucrose test in early identification of both acute and chronic gastric mucosal damage irrespective of the presence of any symptoms and to define the efficacy of a single daily PPI administration in protecting gastric mucosa in patients requiring long term treatment with NSAIDs.

MATERIALS AND METHODS

Patients

Seventy-one consecutive outpatients (38 females, 33 males, mean age 53 years, range 26-78 years) with chronic use of NSAIDs (ASA, diclofenac, celecoxib) for rheumatic diseases (rheumatoid arthritis, osteoarthritis) were enrolled in the study. None of them consumed gastroprotective agents (PPIs, H₂-blockers, misoprostol).

All patients were asymptomatic at baseline and had no previous history of peptic ulcer, gastroesophageal surgery or malignancies.

Sucrose permeability test (40 mg of sucrose in 100 mL of water) was performed at baseline. The patients were divided in group A (31 patients receiving 40 mg pantoprazole daily) and group B (40 patients not receiving PPI therapy). Sucrose test was repeated 2, 4, and 12 wk after follow-up (Figure 1).

The patients gave their informed consent according to the principles of the Helsinki Declaration. Symptoms were recorded according to the presence of mild dyspepsia as previously defined (score 0 = absence; score 1 = mild dyspepsia)^[11].

Sucrose test

To evaluate gastric permeability, the subjects were requested to ingest 40 g sucrose in 100 mL of water after fasted for 8 h. Urine samples were collected in the following 6 h into a container with the addition of 2 mL of 20% chlorhexidine to prevent bacterial degradation. No food or liquid intake (except a glass of water) was allowed during urine collection. Urine samples were refrigerated and taken to the laboratory within 24 h. An aliquot was stored at -20°C for subsequent analysis.

Sucrose assay was based on its hydrolysis by β-fructosidase to glucose and fructose. Glucose was measured by the hexokinase method. The concentration of the reduced nicotinamide adenine dinucleotide phosphate (NADP) was measured at 340 nm. Results were expressed as the percentage of excretion of the ingested dose of sucrose and the normal range was < 0.15% sucrose.

Statistical analysis

Data were reported as mean ± SD. Values of sucrose test at baseline and after 2 and 4 wk in group B were evaluated with Wilcoxon signed-rank test. Comparison between sucrose test values in groups A and B was made with Mann-Whitney test. Statistical Program for Social Sciences (SPSS Inc., Chicago, Illinois 60606) was used for calculations. *P* < 0.05 was considered statistically significant.

RESULTS

At baseline all the patients showed a mean sucrose value of 0.10% ± 0.08%. Two weeks later, all the patients underwent an additional sucrose test which showed a mean value of 0.13% ± 0.08% in group A and 0.19% ± 0.06% in group B. At wk 4 sucrose test showed 0.12% ± 0.07% in the pantoprazole group and 0.24% ± 0.07% in the other group (*P* = 0.000). Sucrose test was then repeated after 12 wk, showing the mean values of 0.13% ± 0.10% in group A and 0.25% ± 0.07% in group B respectively (*P* = 0.000) (Figure 1). At wk 4 the mean value of sucrose

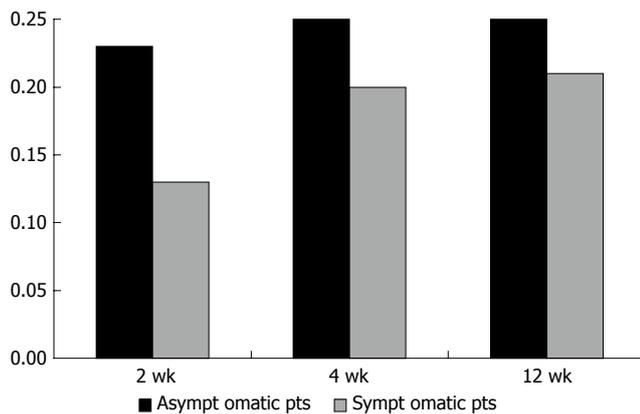


Figure 2 Sucrose test in Group B patients accordingly to their symptoms.

test decreased in 7 patients of group A and in 15 patients of group B. At wk 12 the mean value of sucrose test decreased in 3 and an additional 3 patients of group A and none of group B. Forty-six patients (21 in group A and 25 in group B) completed the follow-up (wk 12).

Regarding the consumption of NSAIDs, the patients on ASA therapy and without gastroprotection showed the mean sucrose values of $0.10\% \pm 0.05\%$ at baseline and $0.17\% \pm 0.06\%$, $0.24\% \pm 0.07\%$, $0.28\% \pm 0.06\%$ at 2, 4 and 12 wk respectively.

In the patients consuming diclofenac and without gastroprotection, the mean sucrose values were $0.08\% \pm 0.03\%$ at baseline and $0.24\% \pm 0.05\%$, $0.31\% \pm 0.06\%$ and $0.30 \pm 0.04\%$ at 2, 4 and 12 wk respectively.

In the patients consuming celecoxib, the mean sucrose values were $0.08\% \pm 0.04\%$ at baseline and $0.04\% \pm 0.05\%$, $0.17\% \pm 0.04\%$ and $0.15\% \pm 0.06\%$ at 2, 4 and 12 wk respectively. The patients with no symptoms accounted for 47.4%, 31.6% and 32.6% after 2, 4 and 12 wk respectively.

In group B, 37.5%, 32% and 28% of the patients remained asymptomatic after 2, 4 and 12 wk respectively. However, their mean sucrose values were $0.13\% \pm 0.08\%$, $0.20\% \pm 0.06\%$ and $0.21\% \pm 0.05\%$ during the follow-up period.

The mean sucrose values in patients complaining of mild dyspepsia in group B were $0.23\% \pm 0.04\%$, $0.25\% \pm 0.08\%$ and $0.25\% \pm 0.07\%$ after 2, 4 and 12 wk (Figure 2).

DISCUSSION

Sucrose test is considered as a useful tool for predicting the presence of clinically significant gastric disease after repetitive exposure to NSAIDs. Furthermore, sucrose test has been shown to be able to detect the differences in both formulation and dose of NSAIDs^[13].

NSAIDs reduce secretion of both bicarbonate and mucus, which are two of the most important gastric mucosal defensive mechanisms. In fact, bicarbonates stimulate cell renewal and repair, while mucus that coats over the mucosal lining provides a substrate for rapid restitution processes^[21,22]. NSAIDs-induced damage allows permeation of macromolecules such as sucrose through the gastric mucosa.

We have previously proposed sucrose test together with serum pepsinogens as useful non-invasive methods to as-

sess histological damages as they show a good relationship with histological findings^[14].

In our study, the patients without PPI therapy showed increasing mean values of sucrose test during the acute period (0.19% after 2 wk and 0.24% after 4 wk) compared to the 0.10% at baseline with a statistically significant difference ($P = 0.000$), suggesting that sucrose test is able to detect gastric mucosal damages in acute ingestion of NSAIDs.

The subjects on chronic therapy showed a stable mean value of 0.25% after 12 wk, without any statistically significant difference ($P = 0.39$). This can be explained with the underlying mechanisms of NSAIDs-induced gastric damage involving both direct irritant action and systemic effect due to the inhibition of cyclooxygenase (COX) activity, which represents their main pharmacological effect. However, some authors suggest that COX-independent effects may be important in inducing mucosal cell apoptosis and microvascular perturbation with subsequent acute lesions^[23].

Several studies have indicated that acute damage is much more widespread than damage observed after several days or weeks, indicating that the gastric mucosa has adaptive mechanisms that compensate for NSAIDs-induced injury^[24]. Although the mechanisms involved in adaptation have not been defined yet, they reflect the capacity of a rapid epithelial repair, which in turn depends on restitution and cell replication^[25].

In our study, the symptomatic patients showed a urinary sucrose excretion above the normal limit, suggesting a gastric damage. However, an alteration in gastric permeability was also undetectable by endoscopy, suggesting that sucrose test represents the only available method to detect "mild to moderate" gastric damages.

In our study, a proportion of patients without gastroprotection revealed higher mean sucrose excretion than normal although they remained completely asymptomatic. These patients had a normal sucrose test at baseline and after 4 wk, but the excretion increased significantly in the following weeks, suggesting that they had mucosal damage. These results indicate an important clinical role of sucrose test which can be used to identify gastric mucosal damages irrespective of the presence of a clinical picture, which is often absent in NSAIDs-induced damage.

Sucrose test could be performed sequentially in high risk patients such as chronic users and rheumatic patients, and could identify early gastric mucosal damages.

The best strategy in preventing NSAIDs-induced damage is still controversial^[13-19]. First, gastrointestinal complications may be avoided by the use of NSAID analgesics when possible. Second, using NSAIDs at their lowest effective dose decreases the risk of complications. Third, medical cotherapy could be used in patients with increased risk of complications. Finally, less injurious NSAIDs such as COX-2-specific inhibitors should be developed to decrease the risk of gastrointestinal events.

Many studies have demonstrated that PPIs give a good protection against both duodenal and gastric ulcers in patients taking NSAIDs^[27,28]. In addition, these drugs are safe and better tolerated as co-therapies than H₂-antagonists or prostaglandin.

Some studies have demonstrated a significant benefit of pantoprazole in preventing NSAIDs-induced peptic ulcers. There are also data regarding the efficacy of pantoprazole in preventing gastric lesions in rheumatic patients receiving long term treatment with NSAIDs. It has been reported that pantoprazole (40 mg daily) is well tolerated and more effective than placebo in preventing peptic ulcer in patients with rheumatic diseases on prolonged therapy with NSAIDs^[28-30].

In our study, the patients on pantoprazole therapy showed lower mean values of sucrose test both in acute and chronic treatment compared with those without gastroprotection, confirming the efficacy of this drug in protecting gastric mucosa. The presence of upper gastrointestinal symptoms was lower in patients receiving pantoprazole during the period of observation.

In conclusion, sucrose test can be used in the follow-up of patients on chronic therapy with NSAIDs. In fact, this method is non-invasive, well tolerated and inexpensive, and can be easily performed sequentially in chronic users of NSAIDs. This approach helps identify high risk patients that should undergo an upper gastrointestinal endoscopy if economic consideration is taken into account.

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Administration of granulocyte colony stimulating factor after liver transplantation leads to an increased incidence and severity of ischemic biliary lesions in the rat model

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Abstract

AIM: Recently it has been reported that granulocyte colony stimulating factor (G-CSF) can induce hypercoagulability in healthy bone marrow donors. It is conceivable that the induction of a prothrombotic state in a recipient of an organ graft with already impaired perfusion might cause further deterioration in the transplanted organ. This study evaluated whether G-CSF treatment worsens liver perfusion following liver transplantation in the rat model.

METHODS: A non-arterialized rat liver transplantation model was employed to evaluate the effect of G-CSF treatment on the liver in a syngeneic and allogeneic strain combination. Study outcomes included survival time and liver damage as investigated by liver enzymes and liver histology. Observation times were 1 d, 1 wk and 12 wk.

RESULTS: Rats treated with G-CSF had increased incidence and severity of biliary damage following liver transplantation. In these animals, hepatocellular necrosis was accentuated in the centrilobular region. These lesions are indicative of impaired perfusion in G-CSF treated animals.

CONCLUSION: G-CSF should be used with caution in recipients of liver transplantation, as treatment might enhance preexisting, undetected perfusion problems and ultimately lead to ischemia induced biliary complications.

INTRODUCTION

In 1975 granulocyte colony stimulating factor (G-CSF) was purified from urine^[1] and serum^[2]. Nowadays G-CSF is in clinical use and is applied routinely for donor conditioning before stem cell transplantation due to its stem cell mobilizing property. It is also used to treat patients who suffer from infections and neutropenia^[3,4] and has been used successfully in endotoxin induced septic conditions^[5]. Recently G-CSF has been used in immunosuppressed patients, particularly transplant-recipients, suffering from CMV infection and severe neutropenia^[6].

Several clinical studies have evaluated the safety of G-CSF for transplant patients with a special focus on acute cellular rejection^[6,7]. Acute cellular rejection, however, is only one potential threat to the transplanted organ. Decreased organ perfusion can lead to impaired function and even graft failure^[8]. Impaired perfusion can result from ischemia-reperfusion damage or arterial insufficiency secondary to thrombosis at the site of anastomosis.

Recently it was shown that G-CSF can induce hypercoagulability in healthy bone marrow donors^[9,10]. A prothrombotic state when a grafted organ is already compromised by impaired perfusion might exacerbate ischemia and further jeopardize the function of the transplanted organ. This study was designed to establish whether G-CSF treatment aggravates impaired liver perfusion following liver transplantation in the rat model.

MATERIALS AND METHODS

Experimental design

A non-arterialized rat liver transplantation model was used

to evaluate the effect of G-CSF treatment on the liver in a syngeneic and allogeneic strain combination. Outcomes included survival time and liver damage as investigated by liver enzymes in combination with liver histology. Observation time was set to 1 d, 1 wk and 12 wk.

Animals

Female Lew and ACI rats were used as donors and male Lewis (LEW, RT¹) rats (Charles River Wiga GmbH, Germany), as recipients. The body weight of male rats was within 250-350 g (10-14 wk old), that of female rats were within 200-300 g (11-14 wk old). The animals were housed under standard animal care conditions and fed with rat chow *ad libitum* before and after operation. All procedures and housing were carried out according to the German Animal Welfare Legislation.

Administration of G-CSF

Animals in the treatment group received G-CSF (Neupogen 48TM, AMGEN GmbH, Germany) at a dose of 100 µg/kg daily by subcutaneous injection starting 5 d prior to the operation until postoperative d 21.

Surgical procedures of orthotopic liver transplantation

Orthotopic liver transplantation was performed according to the technique described by Kamada^[11]. Inhalation anesthesia with Isoflurane was used during the operation.

First step of the surgical procedure was a transverse abdominal incision. The liver of the donor was freed from its ligaments. The small-for-size liver graft was generated by 70% liver resection that included removing the left lateral lobe, the left medial lobe and the middle lobe. The weight of the resected liver lobes as well as the graft itself were recorded. The liver graft was perfused through the portal vein with chilled Ringer's solution. The organ was preserved at 4°C for a maximum of one hour until placed orthotopically in the recipient's abdomen. The donor suprahepatic vena cava was anastomosed end-to-end with the recipient suprahepatic vena cava using a continuous 7-0 polypropylene suture. The portal vein anastomosis as well as the infrahepatic vena cava anastomosis were performed by pulling the recipient's vein over a cuff which was secured with a circumferential 6-0 silk suture. Biliary continuity was restored by tying the bile duct over a stent (Klinika Medical GmbH, Germany).

Postoperative management

After transplantation animals were placed into clean cages, and were given free access to tap water and rat chow *ad libitum*. Animals were observed daily. Body weight loss and general condition including activity, jaundice and bleeding from eyes or nose were recorded. Animals with deteriorating general condition indicated by a weight loss of more than 30% were sacrificed and were classified as dying during the observation period.

Sampling procedures

After sacrifice a full autopsy was performed. Gross findings were documented using a digital camera (Nikon Coolpix 995). Blood was obtained from the infrahepatic

Table 1 Histologic damage score of the liver after liver transplantation

Single cell necrosis	0: No necrotic hepatocyte in 5 HPF (40 X) 1: 1-10 in 5 HPF 2: > 10
Confluent necrosis	0 1: Small in size and number 2: Large size and /or large number
Hepatic mitosis	0: No mitotic hepatocyte in 5 HPF (40 X) 1: 1-10 in 5 HPF 2: > 10 (extensive)
Bile duct inflammation damage	0: No inflammation; 1: A minority of the ducts are cuffed and infiltrated by inflammatory cells 2: More than an occasional duct showing degenerative changes or focal degenerative changes; Most of the ducts infiltrated by inflammatory cells 3: Most of the ducts showing luminal disruption, most of the ducts infiltrated by inflammatory cells
Ductular proliferation	0: None 1: Minimal (small proliferation in a minority of portal tracts) 2: Mild (most portal tracts but not involving the lobular parenchyma) 3: Moderate (all portal tracts and extending along the fibrous septa) 4: Severe (extending along the portal tracts and also slightly involving the lobular parenchyma) 5: Very severe (diffuse proliferation in the lobular parenchyma)
Fibrosis	0: None 1: Fibrosis slightly extending portal tracts 2: Fibrosis extending portal tracts with incomplete septa 3: Fibrosis with complete septa bridging portal to portal tracts 4: Incomplete (focal) or complete cirrhosis
Activated Kupffer cells	0: No activated Kupffer cells 1: Activated Kupffer cells
Eosinophilic globuli	0: No eosinophilic globuli 1: 1%-5% of all hepatocytes 2: > 5%
Small vacuolar transformation of the cytoplasm	0: No small vacuolar transformation of the cytoplasm 1: 1%-30% of all the hepatocytes 2: > 30%

vena cava. Tissue samples (liver, bile duct) were fixed in 4% buffered formalin. Paraffin embedding was performed using standard techniques (Tissue Processor TPC15, Medite Inc.). Paraffin sections (4 µm) were stained with Hematoxylin-Eosin (HE) and Elastic von Gieson (EvG).

Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), Alkaline phosphatase (AP) and Bilirubin were measured with an Automated Chemical Analyzer (ADVIA 1650, Bayer AG, Germany).

Histological evaluation of grafts

Damage to the liver was assessed histologically using a semiquantitative scoring system (Table 1). The scoring

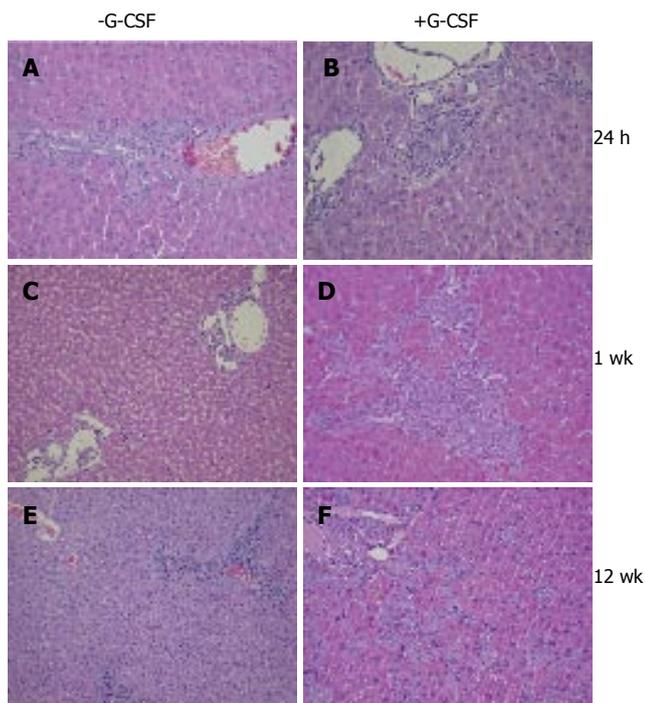


Figure 1 Histology of rat liver after 24 h (A, B), one week (C, D) and 12 wk (E, F) with and without G-CSF treatment showing varying degrees of inflammation and bile duct proliferation. HE; A, D, F 200 x; B 300 x; C, E 100 x.

system included assessment of the portal and lobular inflammatory infiltrate, the extent of hepatocellular necrosis and signs of unspecific hepatocellular damage like a fine vacuolar transformation of the cytoplasm of hepatocytes. The number of mitotic figures and of eosinophilic globular structures in the cytoplasm of hepatocytes were counted. The extent of bile duct inflammation, regenerative changes of biliary epithelial cells and the number of ductules was reported.

The liver damage score was calculated as sum of the scores of all parameters, and the bile duct damage score was calculated as sum of bile duct inflammation and ductular proliferation score.

Data analysis and statistics

Data analysis and statistical procedures were performed using SPSS 10.0 (SPSS Inc. USA). All graphs were generated using *Sigmaplot* 7.0 (SPSS Inc. USA).

The survival rate was depicted as Kaplan-Meier curve. Survival rates in different groups were tested using the *log rank* test. Biochemistry results and damage scores among different groups were tested using the *Mann Whitney Rank Sum* test.

RESULTS

Normal rats

Normal rats treated with G-CSF ($n = 6$) for 5 d showed a mean leucocyte count of 22 neutrophilic cells per nl compared to 15 per nL without G-CSF treatment, indicating the biologic activity of human G-CSF in rats. An isolated increase in the blood level of alkaline phosphatase was detected in 1 out of 6 animals. ALT, AST

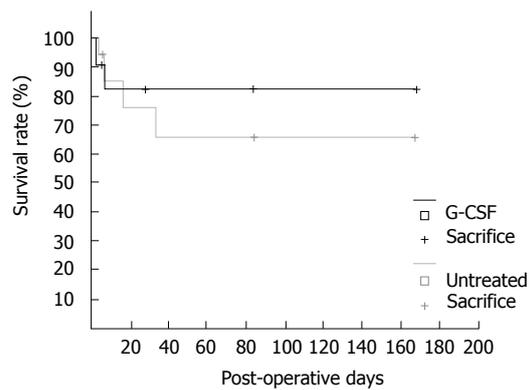


Figure 2 Kaplan-Meier chart of rats after liver transplantation with and without G-CSF treatment.

Table 2 Blood levels of ALT, AST and AP in rats after liver transplantation with and without G-CSF treatment

Group	24 h		1 wk		12 wk	
	Untreated	G-CSF	Untreated	G-CSF	Untreated	G-CSF
ALT (u/L)	616 ± 194 ¹	418 ± 73 ¹	192 ± 49	148 ± 81	92 ± 84	49 ± 24
AST (u/L)	344 ± 204	204 ± 48	96 ± 81	59 ± 28	35 ± 15	24 ± 13
AP (u/L)	141 ± 22 ²	455 ± 99 ²	453 ± 147	449 ± 418	473 ± 543	221 ± 68

¹Significant difference between two groups ($P < 0.05$, *t*-test); ²Significant difference between two groups ($P < 0.001$, *t*-test).

and bilirubin were within their normal range.

Histological evaluation revealed no pathologic changes; especially no alteration of the bile ducts or hepatocellular necrosis.

Rats after syngeneic liver transplantation

In both the G-CSF treated and untreated groups at 24 h, the liver macroscopically showed slight edema in the hilar region. Histologically a granulocytic infiltrate was found surrounding large hilar intrahepatic bile ducts in 50% of all G-CSF treated rats whereas in untreated rats hardly any inflammatory infiltrates in the large hilar intrahepatic bile ducts was detected (Figure 1).

Alkaline phosphatase was significantly higher in the G-CSF group compared to the untreated group (Table 2) indicating a more pronounced damage to the biliary system in the G-CSF treated animals.

Blood levels of liver enzymes were significantly lower in the G-CSF treated group compared to the untreated group (ALT: G-CSF treated group: $418.67 \pm 73.08 \mu\text{L}$ vs untreated group: $634 \pm 195.86 \mu\text{L}$, $P = 0.041$), indicating a reduced number of damaged liver cells in G-CSF treated animals.

In both animal groups morphologic signs of minor hepatocellular damage like small vacuoles in the cytoplasm and a low number of single cell necrosis were visible.

In the treated group hepatocellular damage was mainly located in the centrilobular region. In contrast this distribution of hepatocytic injury was only observed in 2/6 animals of the untreated group. The other animals presented with single cell necrosis throughout the liver

lobule.

In the one week group with G-CSF treatment all animals survived. Survival rate without G-CSF treatment was 90% (Figure 2).

Upon gross examination, signs of limited inflammation around the common bile duct was found in all animals.

AST and ALT levels in both groups were elevated. Compared to the levels reached 24 h postoperatively, a decrease was observed, indicating recovery (Table 2). Two animals in the treatment group showed a massive increase in the AP-levels indicating severe damage to the bile ducts, whereas the other animals were within the normal range. In the untreated group, three out of six animals showed a slight increase in the AP-levels.

Histologically signs of damage to the biliary epithelial cells were visible in animals with and without G-CSF treatment. After G-CSF treatment rats showed a more pronounced proliferation of small biliary ducts suggesting a bile outflow problem.

Biliary epithelial cells showed signs of regenerative activation indicating repair of cellular damage rats with and without G-CSF treatment. Besides mitotic figures the biliary epithelial cells showed an increased nuclear polymorphism and nuclear enlargement leading to a shift of the nuclear to cytoplasmic ratio. Crowding of the biliary epithelial cells was visible. Nuclei did not show basal orientation. Furthermore signs of ongoing damage to the biliary duct were visible. In the cytoplasm of biliary epithelial cells vacuoles were discernible. A very low number of necrotic biliary epithelial cells could be detected. In the vicinity of bile ducts inflammatory cellular infiltrates were visible.

Biliary outflow obstruction is known to induce proliferation of biliary ducts leading to an increase in the number of bile ducts in the portal tracts. Proliferation of bile ducts was more pronounced in animals that underwent G-CSF treatment (Figure 1D). In one rat, the proliferated bile ducts extended into the parenchyma of the liver. In another animal a massive "biliary transformation" of the liver was observed. The biliary duct damage score was slightly higher in G-CSF treated animals at week one, but at this time point the difference did not reach statistical significance ($P = 0.074$, Mann-Whitney test).

Survival rate at 12 wk post operation was 88% in the untreated group compared to the treated group that showed a survival rate of only 64% (Figure 2).

Macroscopically concrements and sludge in large bile ducts was found slightly more frequent in G-CSF treated rats (3/6 compared to 2/6).

In the untreated group, black sludge in the bile duct was found in one rat. In another rat, a small yellow particle in the common bile duct was found, which was associated with a cystic dilatation of the bile duct.

In G-CSF treated animals a small amount of black sludge was found in 2 out of 6 rats. A 0.4 cm × 0.4 cm black stone-like particle in a dilated bile duct was found in another rat. Totally, macroscopically visible lesions of the large extrahepatic bile duct were found in 3 out of 6 rats.

Blood levels of liver enzymes and AP were increased but comparison of both groups did not show a statistically significant difference at this time point.

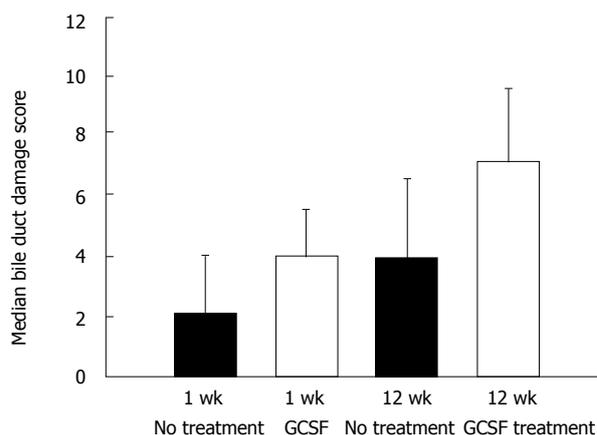


Figure 3 Median bile duct damage score at one and twelve weeks.

Microscopically the number of proliferating small biliary ducts was higher in G-CSF treated animals. The degree of liver cell damage was also higher in G-CSF treated animals. Median bile duct damage score in G-CSF treated animals reached statistical significance compared to rats without G-CSF treatment (Figure 3).

Rats after allogeneic liver transplantation

After full size liver transplantation in the ACI to Lewis strain combination animals died within 9 to 14 d. No difference in survival time was observed between animals with and without G-CSF treatment.

Animals dying spontaneously presented with morphological signs of severe rejection, as expected in this strain combination. Scoring of the histopathology according to the Banff-classification showed no difference in severity between the two groups. Portal tracts were wide and filled with activated lymphocytes and other inflammatory cells. Inflammatory cells extended into the parenchyma of the liver and hepatocellular single cell necrosis was obvious.

In addition inflammatory cells infiltrated small bile ducts that showed signs of damage and destruction. Single necrotic biliary cells and vacuolar transformation of biliary epithelium was the characteristic feature. Regenerative activation of biliary epithelial cells was detected. The number of mitotic figures in biliary epithelial cells was increased and nuclei were not evenly arranged in the vicinity of the basal membrane. In addition nuclear enlargement leading to a shift of the nuclear to cytoplasmic ratio was recorded. In addition to ductitis, there was also an inflammatory infiltrate in the luminal parts of the wall of blood vessels both in the portal tract and in the central regions of the lobules. This endothelitis was accompanied by a regenerative activation and proliferation of endothelial cells and in some vessels, minute fibrin deposits were discernible on the luminal surface.

In the G-CSF treated animals the number of granulocytes seemed to be slightly higher near the bile ducts, whilst hepatocellular single cell necrosis seemed to be slightly more prominent in centrilobular areas compared to rats receiving no G-CSF treatment. Differences were subtle and did not allow for clear discrimination between

Table 3 Signs of a hypercoagulable state after G-CSF treatment in healthy volunteers and in patients with G-CSF producing tumors

Author	n	Observations after G-CSF administration or in patients with G-CSF producing tumors
Topcuoglu <i>et al.</i> , 2004 ^[9]	18	Stimulation of thrombotic factors and increased endothelial markers, such as FVIII and vWF No clinical silent microembolism detected by transcranial Doppler
Sohngen <i>et al.</i> , 1998 ^[13]	25	Hypercoagulable state
LeBlanc <i>et al.</i> , 1999 ^[10]	22	Increased levels of FVIIIc and thrombin Reduced platelet aggregation.
Kuroiwa <i>et al.</i> , 1996 ^[12]	10	Significant increase of platelet aggregation induced by ADP or collagen, thromboxane B2 level and amount of thrombin-antithrombin III complex.
Canales <i>et al.</i> , 2002 ^[32]	20	Significant increase in F1 + 2 and D-dimer Significant decrease of antithrombin and protein C activity Significant increase of vWF Slightly significant decrease of angiotensin converting enzyme
Suzuki <i>et al.</i> , 1992 ^[33]	14	Thrombocytosis

the two groups. When slides were observed in a blinded fashion animals could not be clearly attributed to either group.

DISCUSSION

G-CSF is used routinely in conditioning of donors before stem cell transplantations. In donors and healthy volunteers it has been shown that G-CSF induces a prothrombotic state (Table 3). In addition to the increase in prothrombotic factors, enhanced platelet aggregation was also observed, potentially mediated by the G-CSF receptor on platelets^[12]. Therefore, it was suspected that G-CSF might lead to thrombotic complications in patients already at risk for thrombosis. Thus, careful monitoring was recommended for patients with additional risk factors for thrombosis^[13].

Furthermore other pathophysiological alterations induced by G-CSF may impair microcirculation. Microcirculation was disturbed in G-CSF treated rats using a free-flap model to investigate behavior of granulocytes after chemotherapy and G-CSF stimulation^[14]. Leukocyte rolling and sticking was enhanced to a degree that impaired microcirculation. However, this problem became only visible in this free-flap model with its inherent impairment to microcirculation.

Ischemia-reperfusion injury causing an impairment of microcirculation is unavoidable in transplantations^[15-17]. In clinical liver transplantation, impaired arterial perfusion, mostly due to arterial thrombosis in the hepatic artery, is an important cause of graft dysfunction and even failure^[18-20]. In the rat, rearterialization is not essential for the survival of the recipient, but is considered by some authors to enhance liver perfusion and especially perfusion of the biliary tract, resulting in a lower rate of biliary

complications^[21,22]. For this study, a model with a high risk of perfusion related problems was warranted. Therefore, the non-arterialized rat liver transplantation model, as developed by Kamada^[20] was employed.

In rats, subjected to liver transplantations and additional perioperative G-CSF treatment, in the early postoperative period we observed a centrilobular accentuation of necrotic hepatocytes. At the same time we also observed an increased blood level of alkaline phosphatase at 24 h. Alkaline phosphatase can be an indicator of damage to the biliary epithelial cells but an increase in the blood levels of alkaline phosphatase after G-CSF treatment is also described in some patients due to the induction of bone metabolism^[23]. Especially the pronounced increase after syngeneic liver transplantations in rats treated with G-CSF compared to non-operated G-CSF treated animals suggests damage to the biliary tract leading to cholangitis as the underlying reason.

In the animals observed for 12 wk, an increase in the number and severity of biliary complications and accompanying histomorphological damage was found. In particular, the number of proliferating small biliary ducts, which is indicative of a biliary outflow problem, was more prevalent in G-CSF treated rats.

Impaired arterial perfusion of the liver parenchyma leads to variable centrilobular necrosis^[24]. Impaired arterial perfusion of the bile duct leads to damage of the biliary epithelial cells indicated by the release of alkaline phosphatase. Aggressive bile seeping into the surrounding tissue can perpetuate the damage and attract inflammatory cells, causing cholangitis. Especially when larger bile ducts are affected, there can be a functional impairment of smooth muscle cells with loss of motility, which can impair bile outflow. Reduced biliary flow promotes the development of biliary sludge and concretions eventually leading to outflow obstruction. Biliary obstruction induces proliferation of small bile ducts as demonstrated experimentally in a rat liver ischemia model^[25] and after bile duct ligation^[26].

If this combination of morphological alterations, centrilobular hepatocellular necrosis and cholangitis with or without proliferation of small bile duct is present, as it is in our experiment, an underlying perfusion problem must be assumed. However, it might be difficult to achieve a diagnosis based on histomorphology alone since severe additional confounding alterations like rejection can be superimposed. In the allogeneic strain combination rejection was partially masking perfusion related morphologic alterations. Thus, a perfusion associated problem could only be suspected upon a detailed analysis.

Clinical studies performed to document the safety of G-CSF in transplant patients did not reveal relevant side effects in most studies. G-CSF is known to restore the compromised immune system in neutropenic patients by increasing the number of circulating granulocytes, thus it was suspected that the rejection rate might increase. In the published clinical trials^[6,7,27] an influence on rejection rate or severity was not reported, which was also confirmed in our study. Other problems seem to be out of focus in these studies. In a large clinical trial analyzing potential adverse effects of G-CSF in liver transplanted

patients^[7] not a single case of biliary complications was reported. Biliary problems are rather common after liver transplantations. The complete absence of any biliary problem in such a large cohort of 286 patients is rather surprising.

G-CSF-related histomorphological alterations of the liver were reported in studies, where patients were exposed to high levels of circulating cytokines. Suzuki^[28] reported on a small number of patients with endocrine tumors producing G-CSF as well as IL-1 and IL-8, who presented with liver dysfunction and fever in addition to marked leukocytosis. Biochemical examinations revealed high serum enzyme levels of the biliary system in contrast to normal or slight increases in transaminase levels in all patients studied. Three common pathologic changes of the liver were found: focal necrosis associated with neutrophil infiltration in the centrilobular zones^[1], fibrous change and enlargement of the portal area associated with neutrophil infiltration^[2], and intrahepatic cholestasis^[3]. The same pathologic changes, except for cholestasis, were observed in the liver of mice transplanted with G-CSF-producing cell lines (KHC287 or CHU-2)^[29]. Thus the pattern of liver lesions consisting of an impairment of bile ducts and centrilobular necrosis of hepatocytes was similar to morphologic changes observed in our experiment.

But on the other hand a beneficial effect of G-CSF on the liver after extended liver resection and toxic liver damage has been described^[30,31]. Yannaki observed an accelerated recovery and improved survival after liver injury and attributed this effect to the promotion of endogenous repair programs^[9], and not to stem cell transdifferentiation, as discussed recently. In the experiment presented here, the lower serum levels of AST and ALT 24 h after liver transplantation could be attributed to this beneficial G-CSF effect. In treated rats, hepatocyte necrosis was only observed in the vicinity of central veins, whereas single cell necrosis was distributed throughout the liver lobule in the untreated group, accompanied by a significantly higher release of liver enzymes. This distribution pattern of single cell necrosis gives rise to the speculation, that G-CSF had a beneficial affect on hepatocytes undergoing ischemia reperfusion injury, but had a detrimental effect on perfusion leading to centrilobular necrosis of hepatocytes and necrosis and vacuolization of biliary epithelial cells.

This contradictory effect contributes to the blurring clinical picture, which hinders one from reaching definitive conclusions from these studies. Perfusion related changes are rather subtle, thus it is necessary to give special attention to this problem, which does not always happen, neither in daily clinical routine nor in clinical studies as mentioned before^[7].

CONCLUSION

G-CSF should be used with caution in liver transplanted patients, as treatment might enhance preexisting, undetected perfusion problems and ultimately lead to ischemia induced biliary complications.

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RAPID COMMUNICATION

Usefulness of duodenal biopsy during routine upper gastrointestinal endoscopy for diagnosis of celiac disease

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Abstract

AIM: To describe the trend in duodenal biopsy performance during routine upper gastrointestinal endoscopy in an adult Spanish population, and to analyze its value for the diagnosis of celiac disease in clinical practice.

METHODS: A 15 year-trend (1990 to 2004) in duodenal biopsy performed when undertaking upper gastrointestinal endoscopy was studied. We analysed the prevalence of celiac disease in the overall group, and in the subgroups with anaemia and/or chronic diarrhoea.

RESULTS: Duodenal biopsy was performed in 1033 of 13 678 upper gastrointestinal endoscopies (7.6%); an increase in the use of such was observed over the study period (1.9% in 1990-1994, 5% in 1995-1999 and 12.8% in 2000-2004). Celiac disease was diagnosed in 22 patients (2.2%), this being more frequent in women than in men (3% and 1% respectively). Fourteen out of 514 (2.7%) patients with anaemia, 12 out of 141 (8.5%) with chronic diarrhoea and 8 out of 42 (19%) with anaemia plus chronic diarrhoea had celiac disease. A classical clinical presentation was observed in 55% of the cases, 23% of the patients had associated dermatitis herpetiformis and 64% presented anaemia; 9% were diagnosed by familial screening and 5% by cryptogenetic hypertransaminasaemia.

CONCLUSION: Duodenal biopsy undertaken during routine upper gastrointestinal endoscopy in adults, has been gradually incorporated into clinical practice, and is a useful tool for the diagnosis of celiac disease in high risk groups such as those with anaemia and/or chronic diarrhoea.

INTRODUCTION

Celiac disease (CD) is a frequent immune-mediated enteropathy of worldwide distribution. It affects both children and adults and has a heterogeneous clinical presentation^[1]. Even though we currently possess sensitive and specific serological methods, the duodenal biopsy continues to be the gold standard for the diagnosis of CD^[2]. Knowledge of the diverse forms of presentation of CD, together with a high index of clinical suspicion and an improvement in the accessibility to endoscopy units have made it possible, in some geographical areas, that the number of diagnosed celiac patients approximates the number of patients estimated from population screening studies^[3].

Endoscopic duodenal biopsy is a diagnostic tool in the management of patients with chronic diarrhoea and ferroperenic anemia^[4,5], these being frequent manifestations of CD^[1]. Several studies have been designed in order to know the prevalence of CD among those with anemia or diarrhoea^[6-13]. However, the usefulness of duodenal biopsy during upper gastrointestinal (GI) endoscopy for diagnosis of CD is less well known in daily clinical practice^[14].

In Spain, the prevalence of CD in the general population is similar to other European countries (1/118 to 1/389)^[15-17], we do not however possess data on the performance of duodenal biopsy in adults in clinical practice. The aims of the present study were to obtain information on the evolution of the practice of duodenal biopsy in adults over a 15-year period in a Spanish Digestive Endoscopy Unit and also to study the efficacy of such for the diagnosis of CD in high risk groups

of patients, such as those with anemia and/or chronic diarrhoea. The clinical characteristics of celiac patients diagnosed throughout the study are also described.

MATERIALS AND METHODS

Patients

The Gastroenterology Unit of Hospital Valle del Nalón (Asturias, Northern Spain) is the reference for an adult population of 75 212 people (27% > 60 years). All upper GI endoscopies were performed by three gastroenterologists (RS, F-RE and NR) and duodenal biopsies (3-5 samples per patient) were evaluated by the same pathologist (DF). A special interest in CD began in 1997, when an epidemiological study was undertaken on the prevalence of the disease in the general population of this area^[15]. In clinical practice, the access to Digestive Endoscopy Unit is not open to primary care and serological testing for CD has always been available (antigliadine, antiendomysium and/or antitransglutaminase, according to the period analysed). From 1998, the genetic study of HLA-DQ2/DQ8 has also been available.

Study design

Using the computerized register of all upper GI endoscopies performed between 1990 and 2004 (13 678), we created a database of all patients who had undergone a duodenal biopsy (1033). After excluding 12 explorations in which it was not possible to reach a histological diagnosis due to technical problems and another 12 which were performed in already known celiacs, the total sample analysed was 1009 explorations. The variables included for study are as follows: year when duodenal biopsy was undertaken, age and gender of patient, indication for upper GI endoscopy and the final histological diagnosis.

We studied the evolution over time of the performance of duodenal biopsy during the upper GI endoscopies; in some cases the endoscopic exploration was made exclusively in order to perform duodenal biopsies, since a high index of suspicion of CD previously existed (for example, positive serology or malabsorption syndrome). However, in the majority of the cases, it was the endoscopist who decided, at the time of exploration, whether or not to take duodenal biopsies, when faced with the finding of anomalies in the intestinal mucosa or the presence of certain symptoms or analytical changes (diarrhoea, anemia, iron deficiency, *etc*).

We analysed those cases diagnosed as celiacs; the enteropathy was described according to the Marsh' classification (modified by Oberhuer)^[18] and a response to a gluten-free diet, was used as the diagnostic criteria for CD. We studied the frequency of CD, according to the main indication for performing the duodenal biopsy. The subgroup of patients with anemia ($n = 514$) included those in which the indication for endoscopic study was the presence of ferropenic anemia, microcytic anemia or iron and/or folic acid deficiency; patients with anemia due to vitamin B12 deficiency or those in whom the anemia was associated with signs or symptoms of upper GI bleeding, were excluded from the analysis. The subgroup of patients

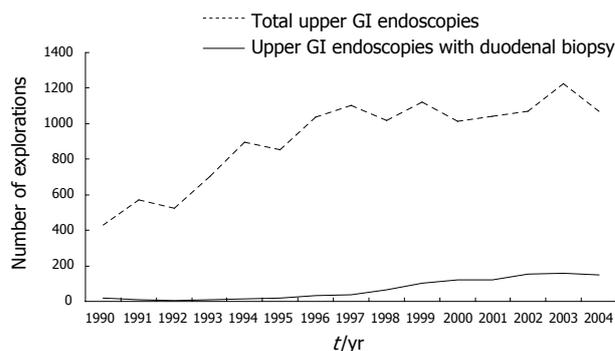


Figure 1 Fifteen-year evolution of the performance of duodenal biopsy during routine upper gastrointestinal endoscopy in an adult Spanish population.

with diarrhoea ($n = 141$) included those with a picture of chronic diarrhoea (> 4 wk) in whom a possible origin was suspected in the small intestine.

The clinical, serological and genetic characteristics of patients diagnosed with CD over the study period are presented in this paper.

Statistical analysis

Data are expressed as percentages or absolute number for categorical variables and medians and ranges for continuous variables. The Chi-square test was used to test for differences between groups of patients with regard to their clinical and demographic features. Multivariate logistic regression analysis was performed to describe the independent association of variables, and the output was converted into adjusted likelihood ratios. A stepwise (forward) selection procedure was employed in order to prevent exclusion of important variables from the model, as a result of mutual correlations. The covariables introduced in the model were age, gender, anemia, diarrhoea, and year of performance of duodenal biopsy (stratified into three periods). The relationship between CD and the above variables was quantified as odds ratios (OR) and 95% confidence intervals are provided. All statistical analyses were performed using MedCalc[®], Ver. 7.4.4.1 (MedCalc Software). A two-tailed $P < 0.05$ was considered to be statistically significant.

RESULTS

Temporal evolution of duodenal biopsy during the study period

From 1990 to 2004, duodenal biopsy was performed in 1033 out of a total of 13 678 upper GI endoscopies (7.4%). Figure 1 represents the 15-year evolution of the performance of duodenal biopsy in the final sample evaluated (1009); when a division was made into three consecutive periods of five years: 1990-1994, 1995-1999 and 2000-2004, we found a significant increase in the percentage of endoscopic explorations in which duodenal biopsy was performed: 58 out of 3121 (1.9%) in the first period, 258 out of 5128 (5%) in the second period, and 693 out of 5405 (12.8%) in the third (1.9% *vs* 5%, $P < 0.0001$; 5% *vs* 12.8%, $P < 0.001$).

Table 1 Prevalence of celiac disease in 1009 duodenal biopsies performed during upper gastrointestinal endoscopy

	Number	CD <i>n</i> (%)	95% CI
Study population	1009	22 (2.2)	1.4-3.3
Period:			
1990-1994	58	2 (3.4)	0.6-13.0
1995-1999	258	8 (3.1)	1.5-6.3
2000-2004	693	12 (1.7)	0.9-3.1
Gender:			
Female	600	18 (3)	1.8-4.8
Male	409	4 (1)	0.3-2.7
Age (yr):			
14-34	136	3 (2.2)	0.6-6.8
35-54	309	11 (3.6) ^b	1.9-6.5
55-74	365	7 (1.9)	0.8-4.1
≥ 75	199	1 (0.5)	0.03-3.2
Patient subgroups:			
Anemia	514	14 (2.7)	1.6-4.6
Diarrhoea	141	12 (8.5) ^d	4.7-14.7
Anemia plus diarrhoea	42	8 (19) ^f	9.1-34.6

^b*P* < 0.01 (35-54 yr vs ≥ 75 yr); ^d*P* < 0.01 (diarrhea vs anemia); ^f*P* < 0.0001 (anemia plus diarrhoea vs anemia).

Up to 1997 a duodenal biopsy was not indicated by positive serology for CD and up to 1999, this was not made in first-degree relatives of celiac patients. Duodenal biopsy was performed in 7.7% of the patients with anemia as the main indication for upper GI endoscopy during the first period, in 21% during the second, and in 73% during the third period.

Prevalence of celiac disease

The total group included 600 females and 409 males with a median age of 60 years (range 14-93 years), without significant differences in gender. Twenty two patients were diagnosed with CD, which accounted for 2.2% of the duodenal biopsies performed; in 6 subjects (27%) the duodenal biopsy was indicated after knowing the presence of positive serology for CD, while in the 16 remaining patients (73%), this was performed according to the clinical suspicion of CD (based on the presence of chronic diarrhoea, non-specific digestive symptoms, *etc*) or by the finding of persistent analytical changes (anemia and/or iron deficiency). The prevalence of CD in the overall group and also in the subgroups of anemia and/or chronic diarrhoea is shown in Table 1.

In the logistic regression analysis, only the presence of anemia (OR 2.82; 95% CI 1.11-7.14) and diarrhoea (OR 10.38; 95% CI 4.22-25.50) were risk factors for CD. The clinical and immune-histological characteristics of the celiac patients diagnosed in the study are shown in Table 2. No differences were observed in the frequency of classical presentation between patients diagnosed before or after 1999 (50% versus 58%, respectively). A gluten-free diet was strictly followed-up in 20 of the 22 adult celiac patients diagnosed.

Table 2 Clinical and immune-histological characteristics of celiac patients

Gender	
Female	18 (81.8%)
Male	4 (18.2%)
Age (yr)	50 (19-77)
Clinical characteristics	
Malabsorption syndrome	12 (54.5%)
Anemia	14 (63.6%)
Dermatitis herpetiformis	5 (22.7%)
Abnormal liver function test	7 (31.8%)
Celiac relatives	2 (9.1%)
Histology (Marsh type):	
I	3 (13.6%)
II	1 (4.5%)
III	18 (81.8%)
Serology ¹	
AGA	13 (86.7%)
EMA	17 (89.5%)
TTG	8 (88.9%)
HLA-DQ2 ²	18 (100%)
Autoimmune associated diseases ³	2 (9.1%)
Mortality ⁴	2 (9.1%)

n (%) are shown for categorical variables, while the median (range) is shown for age. ¹Serological analysis was performed using antigliadin (AGA) in 15, anti-endomysium (EMA) in 19 and tissue-trans-glutaminase antibodies (TTG) in 9 patients; ²Genetic study was performed in 18 patients; ³Addison disease (1) and juvenile arthritis (1); ⁴Stroke (1) and non-Hodgkin lymphoma (1).

DISCUSSION

This study shows the changes observed over a period of 15 years (1990-2004) in the performance of duodenal biopsy in an endoscopy unit for adults in Spain. At present, the practice of duodenal biopsy during upper GI endoscopies is common. During the period 1990-1994 duodenal biopsy was made in 1.9% of the endoscopic explorations, while during the period 2000-2004 this was 12.8%.

Advancement in the knowledge of CD has led to an increase in the indications for performing duodenal biopsy. It is currently known that mild digestive symptoms (dyspepsia, abdominal discomfort, *etc*), or analytical alterations (anemia, iron deficiency or hypertransaminasaemia), could be some forms of presentation of CD^[1]. We observed that during the first 5-year period, duodenal biopsy was usually made in persons with a classical or malabsorption syndrome, while in the later periods this was mainly performed in subjects with anemia (data not shown). Certain risk groups have been well characterised^[19,20], which has meant that during the upper GI endoscopies practised for whatever reason in persons belonging to these risk groups, duodenal biopsy is made even in the absence of symptoms of enteropathy or without previous serological studies.

CD was diagnosed more frequently in females than in males (3% vs 1%), although gender was not a significant

risk factor for CD, as has been observed in other similar studies^[14]. Only anemia and diarrhoea were found to be risk factors for CD. The prevalence of CD found in patients with anemia (2.7%) is included in the wide range communicated in previous studies (1.8% to 13.7%)^[6-11]; the differences are due mainly to the diverse selection criteria of patients with anemia, since if ferropenic refractory patients are included^[6,7] or subjects in which other causes of anemia have been ruled out^[7], the frequency of CD has been higher; on the other hand, the diagnostic strategy used also influences the results, as some studies have performed serological screening prior to duodenal biopsy^[6,8], while in others a duodenal biopsy has been performed in all cases^[7,11] or only in those in which another cause to justify the anemia was not found during the endoscopic exploration^[10].

We wish to emphasize that our study is based on clinical practice over a long period of time, for which reason various factors could have influenced the results. Thus, the percentage of patients with anemia in whom duodenal biopsy was performed has increased over the periods of time analysed as a consequence of a greater knowledge of the manifestations of the disease; although in the management guides of ferropenic anemia duodenal biopsy is recommended during the endoscopic procedure indicated in order to rule out causes of such in the upper GI tract^[4], this was only undertaken at the end of the period analysed in a percentage of 73% of the cases; the degree of fulfilment of this recommendation in countries such as the USA or the UK is low (10% and 46%, respectively)^[21,22], in spite of the fact that it has been shown that duodenal biopsy for CD in a patient with anemia is a cost-effective approach^[23]. We also observed that there were differences in the degree of implication of the endoscopist in the diagnosis of the disease (data not shown); in this sense, differences in the performance of duodenal biopsy among digestive endoscopic services, in the same country, have been reported^[21].

The generalization of serological testing in the later periods of the study, above all in the primary care level, allowed the selection for duodenal biopsy of celiac patients with anemia as the only manifestation of the disease; on the other hand, 13 out of 14 celiacs with anemia presented specific antibodies for CD (the only negative case had a mild intestinal lesion - Marsh type 1). These data support the strategies of serological screening in subjects with anemia at primary care level^[24,25], in blood donors^[26] or in biochemistry laboratories^[27] as an effective method of increasing the number of patients diagnosed with silent CD.

We found that adults diagnosed with CD had a mainly classical clinical presentation (55% with diarrhoea) and ferropenic anemia (64%), for which reason we believe that there must still be a large part of the celiac iceberg (silent or atypical forms) remaining undiagnosed. The prevalence of CD in the adult general population in our area, has been estimated to be 1/389^[15], which means that only one in nine celiacs could be diagnosed, this being similar to that reported for other geographical areas^[1].

We conclude that duodenal biopsy performed during routine upper GI endoscopy, has been incorporated into

the daily clinical practice in digestive endoscopic services. This has permitted a significant increase in the number of patients diagnosed with CD, although these are probably only a small percentage of the true number of celiacs in the general population. In our area, the existence of anemia and diarrhoea are the most common risk factors associated with the presence of CD. Clinicians should consider CD as a possible cause of unexplained anemia, and gastroenterologists should biopsy the duodenum when exploring patients with iron-deficiency anemia and/or chronic diarrhoea, even if biopsies are not specifically required.

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Gastric cancer in a Caucasian population: Role of *pepsinogen C* genetic variants

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Abstract

AIM: To study the role of an insertion/deletion polymorphism in the *pepsinogen C* (*PGC*) gene, an effective marker for terminal differentiation of the stomach mucosa, in the susceptibility to the development of gastric lesions.

METHODS: The study was performed with 99 samples of known gastric lesions and 127 samples without evidence of neoplastic disease. PCR was employed and the 6 polymorphic alleles were amplified: Allele 1 (510 bp), Allele 2 (480 bp), Allele 3/4 (450/460 bp), Allele 5 (400 bp) and Allele 6 (310 bp).

RESULTS: Our results revealed that Allele 6 carriers seemed to have protection against the development of any gastric lesion (OR = 0.34; $P < 0.001$), non-dysplastic lesions associated with gastric adenocarcinoma such as atrophy or intestinal metaplasia (OR = 0.28; $P < 0.001$) or invasive GC (OR = 0.39; $P = 0.004$).

CONCLUSION: Our study reveals that the Allele 6 carrier status has a protective role in the development of gastric lesions, probably due to its association with higher expression of *PGC*. Moreover, the frequency of Allele 6 carriers in the control group is far higher than that obtained in Asian populations, which might represent a genetic gap between Caucasian and Asian populations.

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Key words: Gastric adenocarcinoma; Pepsinogen C; Polymorphism

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INTRODUCTION

Gastric adenocarcinoma (GC) is a major public health problem worldwide and the third cause of cancer-related mortality in Europe^[1-3]. In Portugal, gastric cancer represents a sixth of all cancer related deaths, with twice the average mortality of European Union and the highest among Western European countries 25/100 000 and 12/100 000 persons × year in men and women, respectively^[4,5]. Portuguese inhabitants show a life-time risk for gastric cancer of approximately 2% (95% CI 1.9-2.1), with half the decline observed at other European countries during the last decade^[6].

Lauren's classification is most commonly used for gastric adenocarcinoma because of its epidemiologic importance^[2,7]. It defines two main morphological types: 'diffuse type' and 'intestinal type'. The latter is characterized by a stepwise transformation from normal mucosa through atrophic gastritis, atrophy, intestinal metaplasia and dysplasia to invasive gastric adenocarcinoma^[2,8]. This type shows a male:female ratio of 2:1 and it has been related mostly to environmental factors such as *H pylori* infection and diet^[2,6].

However, recently several host genetic variations have been regarded as potential risk markers^[9-18]. Pepsinogen is an effective marker of terminal differentiation of the stomach mucosa^[19,20]. Pepsinogen C (*PGC*) is mainly secreted by chief cells of gastric gland, while also by cardiac, pyloric and Brunner glands. Its serum levels have been related with atrophic changes in gastric mucosa and extension of intestinal metaplasia^[21,22]. The *pepsinogen C* (*PGC*) gene, localized on chromosome 6 between regions 6p11-6p21.3, encodes the *PGC*, also known as progastricsin, which is the precursor of pepsin C or gastricsin++^[20]. Taggart *et al* have described an insertion/deletion polymorphism of about 100 bp located between exons 7 and

8^[23-25], which was regarded as a susceptibility marker for the development of gastric adenocarcinoma^[26]. However, the role of this polymorphism has not been completely established and it has never been measured in Caucasian populations.

The aim of this control study was to evaluate the role of the *PGC* polymorphism in the development of GC within a southern European population from the north region of Portugal.

MATERIALS AND METHODS

Patients

A cross-sectional study was performed among healthy individuals without clinical evidence of cancer ($n = 127$ as control group) and consecutive patients with known gastric lesions ($n = 99$), both from the northern region of Portugal attending at the Portuguese Institute of Oncology of Porto (Portugal).

Patients were further divided according to the type of lesions presented upon histopathological diagnosis after endoscopic multiple biopsies. It included patients who displayed lesions as severe as high-grade dysplasia and intestinal type invasive gastric adenocarcinoma ($n = 52$) and patients with non-dysplastic but associated lesions with gastric adenocarcinoma such as atrophy or intestinal metaplasia ($n = 47$), who belonged to a standardized follow-up since 2001.

All individuals included in this study gave their informed consent before their inclusion in the study, according to the Declaration of Helsinki.

Sample collection and DNA extraction

Blood samples were obtained with a standard venipuncture technique using EDTA containing tubes, and the genomic DNA was extracted from the white blood cell fraction of each sample, using a standard salting out protocol^[27].

PGC polymorphism analysis

The analysis of *PGC* polymorphism (insertion/deletion of approximately 100 bp) located between exons 7 and 8 was carried out by polymerase chain reaction (PCR), as described by Yamagata *et al.*^[15]. The PCR reaction was performed with the antisense (*PGCa*: 5'-AGCCCTAA GCCTCTTTTGG-3') and sense primers (*PGCb*: 5'-GGCCAGATCTGCGTGTTTTA-3') in a 50 μ L PCR reaction mixture containing: 1 \times Taq buffer, 1.5 mmol/L of MgCl₂, 0.2 mmol/L of dNTPs, 0.25 μ mol/L of each primer and 1 U Taq DNA polymerase (Amersham Bioscience, USA). Cycling parameters are: 95°C for 5 min for Taq DNA polymerase activation, followed by 35 cycles of denaturation for 60 s at 94°C, annealing for 60 s at 55°C, extension for 60 s at 72°C and a final extension step at 72°C for 7 min.

PCR amplification identified the following products: 510 bp (Allele 1), 480 bp (Allele 2), 460 bp (Allele 3), 450 bp (Allele 4), 400 bp (Allele 5) and 310 bp (Allele 6); which were analyzed by electrophoresis in a 3% MetaP[®] Agarose gel (Cambrex Bio Science Rockland Inc, USA) stained with 5% ethidium bromide (Figure 1).

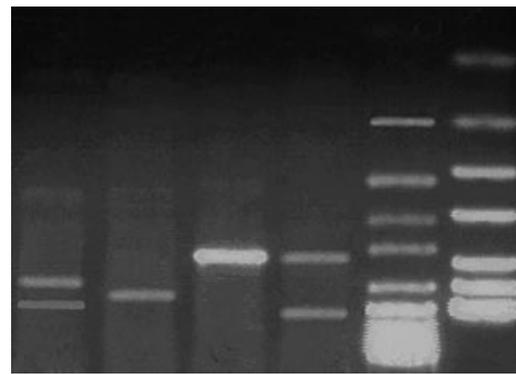


Figure 1 Analysis of *PGC* polymorphism by PCR. M1: 50 bp DNA ladder; M2: pUC DNA ladder; Allele 1 (510 bp); Allele 2 (480 bp); Allele 3/4 (450/460 bp); Allele 5 (400 bp) and Allele 6 (310 bp).

Table 1 Allelic distribution of *PGC* polymorphism according to the type of gastric lesion or its absence n (%)

	Allele 1 carrier	Allele 2 carrier	Allele 3/4 carrier	Allele 5 carrier	Allele 6 carrier
Controls ($n = 127$)	2 (1.6)	31 (24.4)	39 (30.7)	37 (19.1)	92 (72.4)
All cases ($n = 99$)	1 (1.0)	30 (30.3)	33 (33.3)	39 (39.4)	47 (47.5)
AIM ($n = 42$)	-	16 (38.1)	14 (33.3)	16 (38.1)	18 (42.9)
GC ($n = 57$)	1 (1.8)	14 (24.6)	19 (33.3)	23 (40.4)	29 (50.9)

AIM: Atrophy or intestinal metaplasia; GC: Gastric adenocarcinoma.

Variables

The study variables included gastric type of lesion: gastric adenocarcinoma or atrophy or intestinal metaplasia or its absence and *PGC* alleles (1-6).

Statistical analysis

Data analysis was performed using the computer software Statistical Package for Social Sciences - SPSS for Windows (version 11.5). Chi-square analysis was used to compare categorical variables, using a 5% level of significance. Logistic regression was used to estimate odds ratio (OR) and its 95% CI as a measure of the association between *PGC* Allele 6 carrier and risk for the development of gastric lesions.

RESULTS

Allelic distribution of *PGC* polymorphism

The allelic distribution of the *PGC* polymorphism is shown in Table 1. No significant differences between controls and patients with known gastric lesions were observed as far as Alleles 1 to 6 were concerned.

Risk estimates for associated lesions and invasive gastric adenocarcinoma

Table 2 describes the risk estimation for the development of different gastric lesions considering Allele 6 carrier status. Significant differences were found in controls and patients with known gastric lesions concerning Allele 6

Table 2 Association of *PGC* Allele 6 carriers and risk for development of associated lesions and invasive gastric adenocarcinoma

	Allele 6 carrier <i>n</i> (%)	<i>P</i>	OR	95% CI
Controls (<i>n</i> = 127)	92 (72.4)		1.00	Reference
All cases (<i>n</i> = 99)	47 (47.5)	< 0.001	0.34	0.20-0.60
AIM (<i>n</i> = 42)	18 (42.9)	< 0.001	0.28	0.14-0.59
GC (<i>n</i> = 57)	29 (50.9)	0.004	0.39	0.21-0.75

AIM: Atrophy or intestinal metaplasia; GC: Gastric adenocarcinoma; *P*: Pearson Chi-Square; OR: Odds ratio; CI: Confidence interval.

carrier status ($P \leq 0.001$). Moreover, significant differences were also found for Allele 6 carriers when comparing the different types of gastric lesions with healthy individuals ($P \leq 0.001$ and $P = 0.004$).

In addition, the results showed that Allele 6 carrier status had protection against the development of gastric lesions (OR = 0.34; 95% CI 0.20-0.60). This was also true when stratifying the analysis based upon the type of gastric lesions, such as atrophy or intestinal metaplasia (OR = 0.28; 95% CI 0.14-0.59) or invasive GC (OR = 0.39; 95% CI 0.21-0.75).

DISCUSSION

Gastric adenocarcinoma (GC) has distinct geographical distribution with the highest incidence rates in Asian countries, and it remains as the third cause of cancer-related mortality in Europe^[1-3]. Portugal has the highest incidence and mortality rates of Western Europe^[6]. On the other hand, it is well accepted that cancer is a multifactorial disease and that genetic polymorphism may influence the genetic susceptibility to the development of several cancers^[9-18].

Pepsinogen, an effective marker of terminal differentiation of the stomach mucosa, has been used as a serological test for more than 20 years^[21]. An insertion/deletion polymorphism in the *PGC* gene has been described as a susceptibility marker for the development of gastric adenocarcinoma. However, only a few studies have been published on pepsinogen C^[24-26,28,29]. Up to our knowledge, this is the first study within a southern European population, from the north region of Portugal.

Our results suggest that Allele 6 carriers have protection against the development of gastric lesions such as atrophy, intestinal metaplasia or invasive carcinoma. These results are not in concordance with the results in Asian populations (Table 3). By comparison with the results from Asian populations we observed that carriers of this allele were more frequent in our control group, which might represent a genetic gap between Caucasian and Asian populations. However, when we compared the Allele 6 distribution in the Chinese control population, no homogeneous frequencies were reported. Furthermore, the Allele 6 distribution we found within the Portuguese population is not significantly different from one of the Chinese reports^[26].

Table 3 Frequencies of *PGC* Allele 6 carriers within populations

	Population	<i>n</i>	Allele 6 Carriers <i>n</i> (%)
Our Study	Caucasian (Porto, Portugal)	127	92 (72.4)
Ohtaki <i>et al</i> , 1997	Asian (Fukui, Japan)	177	82 (46.3)
Liu <i>et al</i> , 2003	Asian (Shenyang, China)	42	24 (57.1)
	Asian (Zhuanghe, China)	113	69 (61.0)

Even though our study included a small sample of cases, both allelic distribution and risk estimate seemed to exclude a type I error. A cohort study would seem ideal to estimate accurately risks, however, only a few if any cohort studies in this field are published. The recruitment of controls and the follow-up of patients with atrophy and intestinal metaplasia and with the absence of Allele 6 will probably give us more information in the near future.

The role of the *PGC* polymorphism in the carcinogenesis of GC is not clear. It was reported that *PGC* was not only a digestive enzyme, but also a growth factor under strict conditions, whereby the levels of serum expression of *PGC* might play important roles. This *PGC* polymorphism is located between exons 7 and 8, and by the analysis of the genomic sequence (Genome USCS NM_002630; Ref. NM_002630.1) with the Discovery Studio Gene v1.5 (Accelrys Inc), it is possible to identify, within the polymorphic region, an extensive number of TATA-box sequences. TATA-box sequences are extremely important in the activation of gene expression.

We hypothesize that this insertion/deletion polymorphism interferes directly with the number of TATA-box accessible for the activation of *PGC* expression. Usually as many TATA-boxes are available, as many expressions we could achieve. Although, in the presence of a great number of TATA-boxes, altogether in sequence, they might function as a confounder for the transcriptional activation factors. Thus, if a deletion occurs in a site like this, it would stabilize the activation of gene expression, increasing its levels. Evidences of higher levels of *PGC* associated with pre-neoplastic lesions could be correlated with this gene expression stabilization^[22,30].

Although further studies are required to establish the role of this polymorphism in the development of GC, our study reveals that *PGC* Allele 6 (shorter allele) is associated with protection against development of gastric lesions in a Caucasian population.

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Relation between common polymorphisms in genes related to inflammatory response and colorectal cancer

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progression, SNPs may improve appropriate screening for sub-populations at risk.

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Key words: Colorectal cancer; Inflammation; Single nucleotide polymorphisms

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Abstract

AIM: To investigate the association between common single nucleotide polymorphisms (SNPs) in inflammatory response-related genes such as interleukin (IL)-6, IL-8, tumor necrosis factor α (TNF α), peroxisome proliferators-activated receptor γ (PPAR γ), intercellular adhesion molecule-1 (ICAM-1) and the risk of colorectal cancer (CRC) in a group of Greek patients.

METHODS: The study group consisted of 222 CRC patients and 200 healthy controls. Genotyping was performed using allele-specific PCR of PRC-RFLP and the results were confirmed by sequencing. We studied the association of SNPs in the IL-6 (-174G > C), IL-8 (-251T > A), TNF α (-308G > A), ICAM-1 (R241G and K469E), and PPAR γ (Pro12Ala) genes and the risk of CRC.

RESULTS: The IL-6 -174G, R241 and K469 alleles of ICAM-1 were associated with increased risk of CRC (OR = 1.77, 95% CI: 1.34-2.34; OR = 1.83, 95% CI: 1.23-2.72; and OR = 1.35, 95% CI: 1.03-1.77 respectively). The IL-8 and TNF α polymorphisms had no effect. Whereas the PPAR γ Pro12 genotype was associated with increased risk of disease (OR = 1.78, 95% CI: 1.25-2.49).

CONCLUSION: The association between common SNPs in immunologic response-related genes and CRC is reported in the present study. Apart from shedding light on the mechanisms of malignancy initiation and

INTRODUCTION

Colorectal cancer (CRC), whether sporadic or hereditary, is caused by a defined set of molecular events^[1]. A wealth of knowledge has been acquired about the precise molecular events driving CRC formation. Germline mutations in tumor-suppressor adenomatous polyposis coli (APC) genes and DNA mismatch repair (MMR) genes lead to the recognized familial adenomatous polyposis (FAP)-related CRC and the hereditary non-polyposis colorectal cancer (HNPCC), respectively^[1]. These inherited cases account for about 5%-10% of CRC^[1]. Hereditary factors may then contribute to an estimated further 20% of CRC, especially in patients with a strong family history of CRC^[2]. So, up to a quarter of all CRC may occur due to some form of inherited susceptibility to CRC, either in the context of the recognized clinical syndrome or due to germline variants which may carry an increased risk of CRC^[2]. Even without a family history of CRC, 1 in 21 of the population develops the disease, usually later in life^[2]. Sporadic cases of CRC result from somatic hits to the aforementioned genes^[1]. Germline mutation detection is not capable of predicting and screening for sporadic CRC. In other words, excluding inherited types of CRC, the susceptibility of a certain individual to development of sporadic CRC remains largely undetermined.

Since most CRCs arise sporadically, environmental and host immunological factors could significantly

contribute to the initiation and even the progression of this malignancy^[3]. Indeed, colonic cells respond to various malignancies-contributing environmental factors based on the genotype of DNA loci associated with metabolic pathways that relate to various dietary constituents^[3]. Genetic polymorphisms are thought to play a role in determining how individuals respond at the cellular level to various environmental factors^[3].

Immune response undoubtedly has a significant impact on the potential for malignancy, which is highlighted by the clear association between chronic inflammatory conditions and subsequent malignant transformation in the inflamed tissue^[4]. Inflammation favors tumorigenesis by stimulating angiogenesis, damaging DNA and chronically stimulating cell proliferation^[4,5]. The mixture of cytokines that is produced in the tumor microenvironment plays an important role in cancer pathogenesis^[6]. Cancer cells can also respond to host-derived cytokines that promote growth, attenuate apoptosis and facilitate invasion and metastasis^[6]. Importantly, cytokine genetic polymorphisms have recently emerged as determinants of gastrointestinal malignant disease susceptibility and severity^[4]. Although rather limited, evidence already exists linking common polymorphisms in immunologic parameters to CRC tumorigenesis^[5-7].

Bacterial flora keeps the normal colon mucosa in a continuous state of low-grade inflammation, stimulating release of various pro-inflammatory cytokines by the immune cells^[8]. Cytokines activate the NF- κ B transcription factor signal pathway in epithelial and immune cells, leading to up-regulation of interleukin-6 (IL-6) and IL-8^[8]. IL-6 and IL-8 have pro-inflammatory activity in the intestine via the STAT3 intracellular signal pathway^[9]. IL-6 also acts as a potent stimulator of metastasis by up-regulating the expression of adhesion receptors on endothelial cells, such as the intercellular adhesion molecule-1 (ICAM-1)^[10]. The status of a common functional single G > C base exchange in the human IL-6 gene promoter (chromosome 7p21) increases IL-6 levels^[11,12]. It has been found that the A-allele of the -251 T > A SNP in the IL-8 gene is related to higher *in vitro* IL-8 levels after stimulation with lipopolysaccharide and respiratory syncytial bronchiolitis in children^[13].

Tumor necrosis factor α (TNF α) is a pro-inflammatory cytokine whose role has been established in the pathogenesis of rheumatoid arthritis and inflammatory bowel disease (IBD)^[14]. Its binding to two specific receptors sets up signal transduction mechanisms leading to cell apoptosis and gene regulation, via the MAPKinase and NF κ B pathways^[14]. The TNF α pro-cancerous effect has recently been established^[14]. A SNP within the TNF α locus (-308) has been identified in lymphoma patients^[15]. The presence of TNF α -308A allele involved in gene transcription is associated with higher levels of TNF α related to chemotherapy failure and worse overall prognosis^[15].

ICAM-1 is expressed on vascular endothelium and plays a key role in the transendothelial migration of neutrophils and T-cell activation^[16,17]. It functions as a ligand for β 2 integrin molecules present on leukocytes (LFA-1)^[17]. The human ICAM-1 gene is a single-copy gene and contains two polymorphic sites in codons 241 (G/R241; Gly/Arg; exon 4) and 469 (K/E469; Lys/Glu; exon 6)^[18].

Several immunologic disorders, including inflammatory bowel disease are associated with distinct polymorphisms of ICAM-1^[19].

Peroxisome proliferators-activated receptor γ (PPAR γ) is a ligand-activated nuclear transcription factor, which plays a central role in orchestrating gene expression in response to exogenous ligands like other PPARs, such as non-steroidal anti-inflammatory agents (NSAIDs)^[20-22]. Its implication in immunologic mechanisms and carcinogenesis has been strongly speculated. Natural ligands and drug agonists of PPAR γ reduce intestinal inflammation in IBD patients via inhibition of the NF- κ B and STAT3 pathways^[23], while inactivating PPAR γ mutations have been found in sporadic CRC^[24]. PPAR γ has a polymorphism in the coding region 34C > G that results in the aminoacid change of Pro12Ala^[25].

This study focused on the investigation of the aforementioned SNPs as host risk factors for sporadic CRC, using a Greek population cohort.

MATERIALS AND METHODS

Subjects

Two hundred and twenty-two consecutive Greek patients with CRC without previous diagnosis of IBD or any of the known hereditary cancer syndromes (128 males, mean age at diagnosis 66.21 \pm 10.67 years and 94 females, mean age at diagnosis 63.12 \pm 11.52 years) and 200 healthy sex and age matched controls (120 males, mean age 64.73 \pm 15.32 years and 80 females, mean age 60.58 \pm 12.35 years) were genotyped. The vast majority of the studied group consisted of truly sporadic cases (198 patients, 89.2%), while 24 patients (10.8%) reported CRC occurrence in at least one first degree relative. Overall, 35.1% of CRC patients had a family history of malignancy.

Genotypic analysis

DNA was extracted from peripheral blood using the QIAamp blood kit (Qiagen, Germany). To confirm the integrity of DNA, a 430-bp sequence in the human glyceraldehyde-3-phosphatate dehydrogenase (GAPDH) gene was amplified.

The IL-6 polymorphism at position 174 was analyzed by allele specific PCR as previously described^[26]. Briefly, we used primers framing a 347-bp region surrounding the G-174C allele to amplify the genomic DNA isolated from patients and controls by PCR (sense, 5'-TTGTCA AGACATGCCAAGTGC-3'; anti-sense, 5'-CAGAATG AGCCTCAGAGACATCTCC-3'). In addition, each PCR reaction contained two additional primers designed to detect the G-174G allele (anti-sense, 5'-GCAATGACGT CCTTTAGCATCG-3') and another primer designed to detect the G-174C allele (sense, 5'-CCCCCTAGTTGTG TCTTGCCA-3'). We performed multiplex PCR with all the four primers in one tube. PCR products were isolated on 3% agarose gels and visualized with ethidium bromide staining. Individuals with the IL-6-174 C/C genotype were considered low producers and those with the IL-6 -174 G/C or G/G genotype were considered high producers^[27]. Single nucleotide polymorphism-251 T > A in the IL-8 gene was determined using allele specific PCR^[13]. The

allele specific primers were 5'-CCACAATTTGGTGAA TTATCAAT-3' (-251A) or 5'-CCACAATTTGGTGAA TTATCAAA-3' (-251T). The consensus primer was 5'-TGCCCCTTCACTCTGTAAAC-3', giving a PCR product of 336 bp. PCR products were isolated on 2% agarose gels and visualized with ethidium bromide staining.

The A-allele of a single nucleotide polymorphism in the promoter region of the TNF α gene (-308G > A) was determined by PCR-RFLP. The primer sequences used were: forward 5'-AGGCAATAGGTTTGGAGGGCCA T-3' and reverse 5'-TCCTCCCTGCTCCGATFCCG-3', which could amplify a 107 bp sequence. PCR products with a G at position -308 were digested by *Nco* I to give two fragments of 87 bp and 20 bp. Those with an A-allele at position -308 were not digested by *Nco* I as previously described^[28].

Single base polymorphism at codon 241 (R241G) in exon 4 was determined using allele specific PCR as previously described^[29]. We used the primers (5'-GTGG TCTGTTCCCTGGACG-3' and 5'-GTGGTCTGTTCC CTGGACA-3') with the last nucleotide complementary to the allelic variant substitution base on the point mutation in question of the gene ICAM-1, and a common primer (5'-GCGGTCACACTGACTGAGGCCT-3'). The amplified PCR products of 137 bp were analyzed by 2% agarose gel electrophoresis followed by ethidium bromide staining and ultraviolet visualization. The second amino acid polymorphism at codon 469 (K469E) in exon 6 was detected as described by Matsuzawa *et al.*^[30]. The K469E polymorphism was amplified using primers 5'-CCARCGGGGAATCAGTG-3' and 5'-ACA GAGCACATTCACGGTC-3'. The PCR products were identified by enzyme digestion with *Bst* UI which cuts the E469 allele but not the K469 allele.

Exon 2 of PPAR γ was amplified using PCR and the primers G2F (5'-CTGATGTCTTGACTCATGGG-3') and G2R (5'-GGAAGACAACTACAAGAGC-3') as previously described^[31]. The 295 bp PCR product was digested overnight with *Hga* I, which cleaves the Ala allele to generate DNA fragments 178 and 117 bp in size. The DNA fragments were separated on 3% agarose gel.

In all cases, the mutations were confirmed by sequencing analysis using a dye terminator cycle sequencing ready reaction kit (Applied Biosystems), and an ABI 377 automated sequencer. As negative control of the PCR amplifications, we used distilled water instead of genomic DNA and confirmed the fidelity of the reactions.

Statistical analysis

Frequency and susceptibilities of mutations among CRC patients and controls were compared with the chi-square (χ^2) distribution test. Odds ratios (OR) for association were estimated using logistical regression accounting for possible covariates: age, sex and for influence by the remaining genotype loci. Hardy-Weinberg equilibrium for the genotypes was tested using the χ^2 distribution test for the difference in the observed and expected frequencies. All tests were 2-tailed and $P < 0.05$ was considered statistically significant. Inference was aided by SPSS (version 11.0.1, SPSS Inc., Chicago, IL).

Table 1 Tumor parameters in colorectal cancer patients

Parameters	Patients (n)
Tumor location	
Rectum	70
Left colon	104
Right colon	48
Tumor size	
\leq 3 cm	69
> 3 cm	153
Growth pattern	
Ulcerative	89
Protruding	133
Differentiation	
Good	43
Moderate	147
Poor	32
TNM stage	
I	32
II	96
III	68
IV	26

RESULTS

Tumor characteristics are depicted in Table 1. Among the CRC patients, the distribution of the IL-6 genotypes was as follows: GG in 111 patients (50%), CG in 76 patients (34.23%) and CC in 35 patients (15.76%). The distribution of genotypes differed significantly from that in the healthy individuals (Table 2). Additionally, the allele IL-6 -174G was associated with increased risk of CRC, since it was found to be overrepresented among CRC patients (67.1% *vs* 53.5%, $P < 0.0001$, $\chi^2 = 16.35$, OR = 1.77, 95% CI: 1.34-2.34).

Concerning the A allele and AA genotype frequencies of IL-8 at position -251, no significant differences were observed between CRC patients and healthy controls (Table 2). Similarly, no effect of the TNF α (-308G > A) polymorphism was found (Table 2).

Table 2 also summarizes the allelic frequencies and genotypes of the ICAM-1 gene in CRC patient group and controls. No significant differences were observed in the RR (for the R241G) or in the KK (for the K469E) genotype frequencies between CRC patients and controls. Nevertheless, the allelic frequency of both R241 and K469 was significantly higher in CRC patients than in controls ($P = 0.002$, $\chi^2 = 9.24$, OR = 1.83, 95% CI: 1.23-2.72; and $P = 0.031$, $\chi^2 = 4.62$, OR = 1.35, 95% CI: 1.03-1.77, respectively).

As also indicated in Table 2, the C allele (Pro12) of PPAR γ was significantly associated with an increased risk of CRC since it was found to be overrepresented among CRC patients compared to controls (84.7% *vs* 76.5% respectively; $P = 0.001$, $\chi^2 = 9.08$, OR = 1.78, 95% CI: 1.25-2.49). Consequently, the CC genotype appeared as the major risk genotype for CRC ($P < 0.001$, $\chi^2 = 16.43$, OR = 2.01, 95% CI: 1.32-3.09).

When co-carriage rates of the offending alleles (IL-6 -174G, ICAM-1 R241, ICAM-1 K469) were compared

Table 2 Allele and genotype frequencies of the polymorphisms under investigation in CRC patients and healthy controls

		Alleles				Genotypes				
IL-6										
	C	G	G allele frequencies (%)	P; OR (95% CI)	CC	CG	GG	GG genotype frequencies (%)	P; OR (95% CI)	
CRC	146	298	67.1	< 0.0001; 1.77 (1.34-2.34)	35	76	111	50	< 0.0005; 2.10 (1.40-3.16)	
Controls	186	214	53.5		50	86	64	32		
IL-8										
	A	T	T allele frequencies (%)	P; OR (95% CI)	AA	AT	TT	TT genotype frequencies (%)	P; OR (95% CI)	
CRC	186	258	58.11	0.467; 1.10	40	106	76	34.23	0.256; 0.77	
Controls	174	218	54.5	(0.84-1.45)	42	90	64	32	(0.49-1.20)	
TNF α										
	G	A	A allele frequencies (%)	P; OR (95% CI)	GG	GA	AA	AA genotype frequencies (%)	P; OR (95% CI)	
CRC	360	84	18.92	0.265; 1.18	152	56	14	6.31	0.586; 1.28	
Controls	336	64	16	(0.83-1.68)	146	44	10	5	(0.53-3.1)	
ICAM-1 R241G										
	G	R	R allele frequencies (%)	P; OR (95% CI)	GG	GR	RR	RR genotype frequencies (%)	P; OR (95% CI)	
CRC	362	82	18.47	0.002; 1.83	144	74	4	1.8	0.652; 1.50	
Controls	356	44	11	(1.23-2.72)	158	40	2	1	(0.26-8.7)	
ICAM-1 K469E										
	E	K	K allele frequencies (%)	P; OR (95% CI)	EE	EK	KK	KK genotype frequencies (%)	P; OR (95% CI)	
CRC	236	208	46.85	0.031; 1.35	70	96	56	25.22	0.221; 1.33	
Controls	242	158	39.5	(1.03-1.77)	77	88	35	17.5	(0.83-2.12)	
PPAR γ										
	C	G	C allele frequencies (%)	P; OR (95% CI)	CC	CG	GG	CC genotype frequencies (%)	P; OR (95% CI)	
CRC	376	68	84.7	0.001; 1.78	164	48	10	73.8	< 0.001; 2.01	
Controls	306	94	76.5	(1.25-2.49)	118	70	12	59	(1.32-3.09)	

between CRC patients and controls, no statistical differences were found. The distribution of genotypes was consistent with Hardy-Weinberg equilibrium only in IL-8 and ICAM-1 cases ($P > 0.05$). Deviation was observed in IL-6, TNF α , and PPAR γ cases ($P < 0.05$).

No allele frequency difference was observed when sex and age of either patients or controls were taken into account in the statistical analysis. In addition, stratification of cases by site, tumor stage and the rest of the examined histopathological parameters did not reveal any significant association with the studied polymorphisms.

DISCUSSION

The most compelling evidence for the role of inflammation in gastrointestinal (GI) malignancy comes from studies showing that pro-inflammatory cytokine gene SNPs increase the risk of cancer and its precursors. A number of pro-inflammatory genotypes related to known cytokines (IL-1B, TNF α , IL-10) increase the risk of *H pylori*-induced gastric atrophy and gastric cancer^[32,33]. At present, relatively limited information exists on the relationship between colorectal malignancies and cytokine polymorphisms^[5,7,34]. Polymorphisms in the IL-6 gene promoter and the R241 and K469 ICAM-1 polymorphic sites were associated with a significantly increased risk of CRC, whereas the PPAR γ CC genotype and C allele were both related to reduced risk in the present study.

Our results contrast with the findings of Landi *et al*^[5] in terms of the influence of IL-6 SNPs on CRC predisposition, but parallel their results as far as the potential protective role of the PPAR γ Ala12 variant. The researchers con-

cluded that the IL-6-174 C genotype confers an increased risk of CRC^[5], but in our study, a similar outcome was related to the presence of the G allele in the respective gene. The biological role of the substitution 174G > C has not been fully elucidated by functional studies. Although the C-174 allele is associated with both lower and higher levels of IL-6 expression in various conditions^[35-37], one study^[7] has been conducted to evaluate the effect of -174G > C SNP on IL-6 serum levels in CRC patients, and found that the GG genotype and absence of the C allele are related to significantly increased levels of IL-6, particularly in the presence of hepatic metastasis. Accordingly, *in vitro*, macrophages from C-negative subjects produce higher IL-6 levels than those from C-positive subjects^[38]. Moreover, the fact that high levels of circulating IL-6 are observed in patients with different tumor types and the finding of different levels of IL-6 expression in relation to genetic variants suggest that this cytokine is mainly produced by host cells rather than by cancer cells^[7]. We could speculate that the IL-6 G allelic variant might influence the increased cytokine production by immune cells, which in turn induces its stimulatory effect in tumorigenesis. Interestingly as well, Landi *et al*^[5] reported that the C genotype is associated with an increased risk of CRC only in those subjects who do not habitually take NSAIDs^[5]. A possible cause for the conflicts and mismatches, like those observed here and the earlier study in allele and genotype distributions, may be the differences in ethnic backgrounds. The IL-8 A-251 allele's protective role against CRC, as having been demonstrated by Landi *et al*^[5] could not be replicated in our study. It is true that the function of this polymorphism and the role of IL-8 in intestinal inflammation have not yet been

adequately studied^[5].

Previous reports support that there is no significant association between the TNF α -308A allele and CRC development like in our study^[5,39]. The latter possibly precludes any hypothetical involvement of TNF α -308A in CRC tumorigenesis. In contrast to the aforementioned lack of relevance, the examined TNF α SNP has been found to participate in a genetic profile associated with a high risk for gastric cancer^[32,40]. The pro-inflammatory and acid inhibiting properties of TNF α seem to enhance *H pylori* oncogenic or other effects on gastric mucosa^[32,40]. Although a similar extrapolation regarding certain large bowel-colonizing bacteria could be made, such a hypothesis would not be supported based at least on our results. Recent studies on SNPs at the TNF α -308 locus have not found any difference between prostate and breast cancer patients or controls^[41,42]. In contrast and at least for breast cancer, assays for germline SNPs in TNF β instead of TNF α may prove useful, since at least for breast cancer, the TNF β G/G genotype increases the risk of tumorigenesis, while the A allele inhibits that risk^[42].

The present study is the first study investigating ICAM-1 SNPs in CRC and reporting the increased allelic frequency of R241 and K469 polymorphisms in this cancer. Previous studies also reported that both R and K allelic variants are associated with IBD^[16,19,30], which adds to the mounting molecular evidence about the similarities between IBD-associated cancer and sporadic cancer^[8]. Based on ample data concerning the molecular mechanisms involved in colonic mucosal inflammation effects, it is reasonable to speculate that even sporadic cancer might be largely secondary to inflammation^[8]. The normal colon could be viewed as being in a perpetual state of inflammation, where cytokine profiles, glycosylation changes, other inflammatory molecule (i.e. prostaglandins) regulation and mucosa-associated bacteria play a central role^[8]. Adhesion molecule activity may also have a serious impact on immunologic response. R241 and K469 amino acid changes in the ICAM-1 gene may influence its functional role, as both are located on the Mac-1 binding domain and in the immunoglobulin-like domain 5, respectively. The relevance of these specific SNPs could be explained by the functional alteration of the gene product^[19]. Some of the inhibitors of HMG-CoA reductase (statins) have also been shown to bind to the ICAM-1 binding domain of LFA-1^[43]. The regulation of interaction mediated by adhesion molecules may provide a new target for controlling inflammatory and immune responses.

In keeping with Landi *et al*^[5], the PPAR γ Ala12 variant is associated with reduced CRC risk. In our study, the PPAR γ CC genotype (Pro12) had the strongest relative risk for CRC among the examined genes. The protective effect of the Ala12 variant of PPAR γ gene has been recently confirmed by Gong *et al*^[44] in sporadic colorectal adenomas. Similarly the polymorphic allele Ala12 is significantly over-represented in glioblastoma multiform patients, but such an association could not be verified in prostate cancer^[25,45]. The PPAR γ Ala12 is associated with increased tissue sensitivity to insulin, a decrease in insulin plasma level and reduced release of free fatty acids by adipocytes, which in turn are associated with reduced CRC risk^[46,47]. Other plau-

sible mechanisms accounting for this protective effect may lie in PPAR γ involvement in regulation and interactions with other colonic cell functions. Activation of PPAR γ by ligands inhibits the NF- κ B and STAT3 pathways attenuating IBD and related conditions, induces differentiation and apoptosis of CRC cells and decreases DNA synthesis in CRC cell lines^[23,48-50]. The recently investigated transcriptional regulation of the tumor suppressor PTEN gene via PPAR γ may provide a mechanism for phosphatidylinositol 3-kinase (PI-3kinase)-mediated signaling cascades^[51]. Disruption of PPAR γ expression seems to prevent the up-regulation of PTEN leading to resistance of tumor cells (i.e. pancreatic cancer cells) and macrophages to apoptosis^[52]. The latter may open a new dimension in fully understanding the complex mechanisms involved in the interaction of PPAR γ and various tumorigenesis elements.

PPAR γ activation can lead to altered expression of COX-2, while NSAIDs have been reported to be capable of activating PPAR γ ^[21]. NSAIDs can also alter synthesis of eicosanoids that may bind to and act as ligands for PPAR γ through their effects on COX activity^[21,22]. Acetylsalicylic acid causes an approximate halving of risk for sporadic CRC^[53]. In an era of ongoing attempts to identify suitable target population groups for chemoprevention with anti-inflammatory agents, such SNP analysis and mapping of suitable loci may prove invaluable.

It is noteworthy that other immunology-unrelated genes may also carry an increased risk of developing CRC. It was reported that the Harvey ras-1 variable number tandem repeat (HRAS1-VNTR) polymorphism, the methylenetetrahydrofolate reductase (MTHFR) valine/valine polymorphism and missense mutations in CDH1 (E-cadherin) gene all are associated with an increased risk of CRC tumorigenesis^[2]. Apart from shedding light on the mechanisms of sporadic malignancy initiation and progression, SNPs may provide appropriate screening recommendations in intermediate-risk patients, such as those with family history. Only a limited subset of our cohort consisted of such patients and no significant differences were derived in our analysis. Although rather difficult and possibly imprecise during the process of similar studies, taking relevant dietary and lifestyle habits into account is of paramount importance. Simultaneous genotyping and combined analysis of different SNPs in large numbers of patients and controls, stratified by ethnicity, gender and tumor location may make it possible to describe the exact relations between polymorphisms and CRC susceptibility with higher power and may open the way for population-wide genetic screening in the future.

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RAPID COMMUNICATION

Polymorphisms of interleukin-1R receptor antagonist genes in patients with chronic hepatitis B in Iran

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Abstract

AIM: To investigate the relationships between polymorphisms of interleukin-1R receptor antagonist genes and susceptibility to chronic hepatitis B in Iran population.

METHODS: Genomic DNA was extracted from the peripheral blood of 80 patients with chronic hepatitis B (57 males, 23 females) aged 12-77 years (mean 36.1 ± 13.8 years) and 147 normal controls (96 males, 51 females) aged 6-75 years (mean 41 ± 18.7 years) who referred to a liver clinic of Tehran and then subjected to polymerase chain reaction (PCR) amplification. PCR products were resolved on a 3% agarose gel and stained with ethidium bromide.

RESULTS: Only three of the five kinds of polymorphism (2/2, 2/4, and 4/4) were found in this study. The frequencies of 2/2, 2/4, and 4/4 were 12.5%, 17.5%, 70% respectively in chronic hepatitis B patients and 6.8%, 24.5%, and 68.7% respectively in controls. IL-1 R allele 2 was detected in 30% of chronic hepatitis B patients and in 31.3% of controls, while IL-1 R allele 4 was detected in 87.5% of chronic hepatitis B patients and in 93.2% of controls. The frequency of IL-1R alleles 2 and 4 was detected in 21.25% and 78.75% of the patients and 19.04% and 80.96% of the controls, respectively.

CONCLUSION: Our results suggest that the carriage of IL-1R receptor antagonist alleles 2, 4, 6 may not play any role in the development of HBV infection. Large population-based studies are needed to investigate the role of IL-1 polymorphisms in the pathogenesis of developing chronic hepatitis B.

INTRODUCTION

Hepatitis B virus (HBV) infection is one of the most important chronic viral diseases in the world. An estimated 400 million people worldwide are carriers of HBV, and approximately 250 000 deaths occur each year as a consequence of fulminant hepatic failure, cirrhosis, and hepatocellular carcinoma^[1]. According to the last health and disease survey held in Iran in 1999^[2], the prevalence of HBV carriers is 1.7%. Therefore over one million people in Iran are HBV carriers. When HBV is acquired in adulthood, the majority of infections are cleared, with chronic infection occurring in 5%-10% of cases. However, the dynamic interaction of the host inflammatory response to HBV and the subsequent impact of this interaction on the clinical outcome of HBV infection, are not yet fully understood, nor are the underlying mechanisms for persistence of the virus.

Cytokines play an important role in defense against viral infection, indirectly through determination of the predominant pattern of the host response and directly through inhibition of viral replication^[3]. Interleukin (IL)-1 is one of the most pro-inflammatory agents and has a central role in inflammation and destruction^[4]. The most important members of the IL-1 family are the IL-1 α , IL-1 β , and IL-1 receptor antagonists (ra). IL-1ra is an IL-1 natural competitive inhibitor, acting by occupancy of cell surface receptor without triggering signal transduction^[5]. IL-1ra plays a role as an important regulator of inflammation and is currently evaluated in clinical trials. Genes encoding IL-1 are located on the 430 kb region of chromosome 2q13-21, consisting of three homologous genes: IL-1A, IL1B and IL-1ra (IL-1RN)^[6]. Biallelic polymorphisms at positions IL-1A -889, IL-1B -511, and

Table 1 Comparison of IL-1R intron 2 polymorphism between chronic hepatitis B patients and controls

	<i>n</i>	Genotyping (%)						Allele frequency (%)			
		2,2	<i>P</i>	2,4	<i>P</i>	4,4	<i>P</i>	2	<i>P</i>	4	<i>P</i>
IL-1R											
Controls	147	6.8	0.148	24.5	0.225	68.7	0.840	19.04	0.840	80.96	0.148
Chronic hepatitis	80	12.5		17.5		70.0		21.25		78.75	

+ 3953 have been described, all representing a C/T single nucleotide polymorphism (SNP). IL-1RN contains an 86 bp variable number tandem repeat (VNTR) polymorphism in intron 2^[7]. These polymorphisms are located within the regulatory regions of the genes and have a potentially functional importance by modulating IL-1 protein production, and are related with the development of some diseases^[8].

This study was to discuss the relationship between polymorphisms of IL-1R gene and susceptibility to HBV in the Iranian population, and to reveal the correlation between the genotype and phenotype distributions, in order to provide a certain scientific basis for prevention and treatment of chronic hepatitis B.

MATERIALS AND METHODS

Subjects

A total of 80 patients with chronic hepatitis B (57 males, 23 females) aged 12-77 years (mean 36.1 ± 13.8 years) were recruited in a liver clinic of Tehran. The diagnosis of all the patients was confirmed according to the criteria for chronic hepatitis B, and the patients did not have other viral hepatitis. One hundred and forty-seven control subjects (96 males, 51 females) aged 6-75 years (mean 41 ± 18.7 years) were selected in a liver clinic of Tehran (HBsAg negative, anti-HBe negative, and anti-HBc negative). Liver, renal, endocrine and cardiovascular disorders were excluded. There was no statistical difference in sex between case and control groups, and we also saw a statistically significant difference in age between two groups ($P = 0.026$) but it was not clinically important. AST was over 40 U/L in 24.1% of cases and none of controls, and over 40 U/L in 31.6% of cases and none of controls, respectively.

PCR preparation

Two microliter peripheral venous blood was collected in an EDTA tube. Genomic DNA was extracted from peripheral blood leukocytes as previously described^[9]. Each PCR was carried out in 25 μ L reaction mixture containing 100-200 ng genomic DNA, 100 μ mol/L dNTP, 25 mmol/L MgCl₂, 20 pmol/L primers and 1U TaqDNA polymerase (DNA Technology)^[10].

The sequence of the forward primer is 5'-CTCAGCAACACTCCTAT-3'. The reverse primer sequence is 5'-TCCTGGTCTGCAGGTAA-3'. PCR conditions included an initial denaturing step at 94°C for 5 min, followed by 30 cycles at 94°C for 1 min, at 60°C for 1 min, at 70°C for 2 min, and a final extension at 70°C for 4 min. Using this PCR strategy, the common allele (allele 1) generated a 410-bp band (including four copies of an

86-bp repeat). The uncommon alleles generated a 240-bp band (two copies of the same repeat, allele 2), a 500-bp band (five copies of the same repeat, allele 3), and a 325-bp band (allele 4). The PCR products were resolved on a 3% agarose gel and stained with ethidium bromide.

HBV-DNA measurement

Serum HBV-DNA levels in patients with chronic hepatitis B were detected with the real-time fluorescent quantitative PCR method (reagents supplied by Sinagen Co. Ltd.). Results were considered abnormal when HBV-DNA > 200 copies/mL.

Statistical analysis

IL-1R (2-6) allele and genotype frequencies were calculated in patients with chronic hepatitis B and control subjects. Comparison of allele and genotype frequencies between groups, and association of IL-1R polymorphisms with HBV-DNA replication and other variables were examined for statistical significance with chi-square test. Analysis was completed with SPSS11.5. $P < 0.05$ was considered statistically significant.

RESULTS

IL-1R (2-6) allele frequencies were measured. IL-1R 3, 5, 6 were negative in all cases and controls. Therefore we deleted them in our results.

IL-1R genotypes

The intron 2 of IL-1RN polymorphism contained VNTR of 86 bp. There were five alleles in humans, including allele 1 (four repeats, 410 bp), allele 2 (two repeats, 240 bp), allele 3 (five repeats, 500 bp), and allele 4 (four repeats, 325 bp). Only three of the five kinds of polymorphism of IL-1R (2/2, 2/4, and 4/4) were found in this study.

Frequencies of IL-1R genotypes in both groups

The genotype and allele frequencies of IL-1RN in patients with chronic hepatitis B and control subjects were determined, and explored with 2×2 chi-square test (Table 1).

Only three of the five kinds of polymorphism of IL-1R (2/2, 2/4, and 4/4) were found in this study. The frequencies of 2/2, 2/4, and 4/4 were 12.5%, 17.5%, 70% respectively in chronic hepatitis B patients, and 6.8%, 24.5%, and 68.7% respectively in controls. IL-1R allele 2 was detected in 30% of chronic hepatitis B patients and 31.3% of controls, while IL-1R allele 4 was detected in 87.5% of chronic hepatitis B patients and 93.2% of controls. Allele frequency of IL-1R alleles 2 and

4 was detected in 21.25% and 78.75% of the patients and 19.04% and 80.96% of controls respectively.

There were no significant differences between patients and controls in these regards. However, the 2, 2 and 2, 4 and 4, 4 genotypes were not significantly different between patients and controls, which were 6.8% versus 12.5% ($P = 0.148$), 24.5% vs 17.5% ($P = 0.225$), and 68.7% vs 70% ($P = 0.840$) respectively. The genotype distribution in controls was also consistent with the Hardy-Weinberg equilibrium ($P = 0.215$).

DISCUSSION

HBV infection is a major global health problem with an estimated 300 million people chronically infected worldwide^[11,12]. Individuals with an inadequate primary immune response to HBV are at increased risk of developing chronic hepatitis B. Age is the strongest host feature associated with chronic infection with 90% infants and 5%-10% of adults developing chronic hepatitis B after exposure to HBV. In addition, people with the same age, sex and ethnical group are exposed to the same HBV strain, which could cause a broad spectrum ranging from no infection to different clinical outcomes^[13]. These data suggest that host genetic factors are responsible for the clinical outcomes of HBV infection. Clearance of HBV requires a coordinated innate and adaptive humoral and cell-mediated immune response. Cytokines are soluble polypeptide molecules that mediate cell-to-cell communication and regulate the intensity and duration of the immune response. Previous studies have shown that the maximal capacity of cytokine production varies among individuals and correlates with SNP in the promoter region of various cytokine genes^[14,15]. Furthermore, cytokine gene polymorphisms are associated with liver disease severity in patients with viral hepatitis^[16]. In the present study, we compared the distributions of interleukin-1R receptor antagonist gene polymorphisms between patients with chronic B and control subjects.

Interleukin-1R receptor antagonist gene is one of the IL-1 gene family members, and located in the proximal region of chromosome 2q13-21. Different polymorphisms have been described in interleukin-1R receptor antagonist genes, and at least two of them could influence the protein production. The IL-1ra gene is also polymorphic due to a variable number (2-6) of tandem repeats of 86 bp (VNTR) within its second intron^[17]. This polymorphism has been shown to be unambiguously functional at the level of secreted protein, as monocytes from individuals homo- or heterozygous for allele 2 (IL-1Ra A2 1, IL-1RN * 2, 2 repeats) produce significantly more IL-1ra in response to GM-CSF^[18].

Pociot *et al*^[19] reported that IL-1B polymorphisms are correlated with IL-1 β expression. IL-1B allele T carrier has higher productions of IL-1 β than IL-1B allele C carrier. In the present study, genotype distributions and allele frequencies for IL-1B (-511) promoter polymorphisms in patients with chronic hepatitis B and control subjects were not statistically different. Further analysis of the relationship between IL-1B polymorphism and HBV-DNA

replication in patients with chronic hepatitis B showed that IL-1B (-511) genotype CC was associated with HBV-DNA replication.

IL-1Ra is a naturally occurring anti-inflammatory protein, competitively blocks the binding of IL-1 α and IL-1 β type I and type II IL-1 receptors, but exerts no agonist activity, despite sharing 30% amino acid sequence homology with IL-1 β , and 19% with IL-1 α . IL-1ra has been shown to inhibit the effects of IL-1 both *in vitro* and *in vivo*^[20]. There is increasing evidence that IL-1RN polymorphisms are related with susceptibility to individual diseases, including psoriasis, systemic lupus erythematosus, and inflammatory bowel disease^[4]. By the study of interleukin-1R receptor antagonist gene intron 2 polymorphisms, our results suggested that the distributions of interleukin-1R receptor antagonist gene 2/2, 2/4, 4/4 genotype in patients with chronic hepatitis B were not significantly different from those in control subjects. A possible explanation of this result could be provided by the fact that carriage of IL-1R allele of each of these polymorphisms may be related with other production of IL-1 β ^[21], which may augment the production of other cytokines, such as IL-2, IL-6 and TNF- α , and trigger the complex immunological processes to eliminate the virus and its complex. It may be also due to the low sample size of our study (The power of study was 68%).

In summary, our results suggest that the carriage of IL-1R receptor antagonist alleles 2, 4, 6 may not play any role in the development of HBV infection. Large population-based studies are needed to investigate the role of IL-1 polymorphisms in the pathogenesis of developing chronic hepatitis B.

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RAPID COMMUNICATION

Occult hepatitis B virus infection and cryptogenic chronic hepatitis in an area with intermediate prevalence of HBV infection

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Abstract

AIM: To assess the possible role of occult HBV infection in the pathogenesis of chronic hepatitis in Iranian patients.

METHODS: After exclusion of autoimmune, metabolic and viral etiologies, 104 consecutive adult patients with histologic and biochemical features of chronic hepatitis and negative HBsAg were enrolled in the study. Qualitative PCR with a sensitivity of 150×10^3 copies/L, using two primers for Pre-S and core regions was applied to measure presence of HBV DNA in serum of the patients.

RESULTS: All 104 patients completed the study. Qualitative HBV DNA was positive in two patients (1.9%).

CONCLUSION: Occult HBV infection has negligible role in the pathogenesis of cryptogenic chronic hepatitis in Iranian patients.

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Key words: Hepatitis B; Cryptogenic chronic hepatitis; PCR

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INTRODUCTION

Several lines of evidence suggest that hepatitis B virus (HBV) may persist in the serum many years after clearance of hepatitis B surface antigen (HBsAg). These evidences include HBV transmission from liver donors even when HBsAg had been negative and the only positive serologic test was hepatitis B core antibody (HBcAb)^[1-3] and appearance of HBsAg and even overt hepatitis in previously HBsAg-negative patients after cancer chemotherapy or immunosuppression^[4,5]. It appears that low-level HBV infection may be the source of viral reactivation that is unmasked by immunosuppression. It is speculated that these occult infections may cause chronic hepatitis^[6,7], increase the rate of cirrhosis^[8,9] and eventually hepatocellular carcinoma^[10] and may transmit *via* blood transfusion.

The causes of undetectability of HBsAg in serum (in the presence of persistent HBV infection) may include mutations in the pre-S or S regions (mostly in "a" determinant region) of the virus genome^[11-14], decrease in viral load^[15] or insufficient sensitivity of the tests used to detect the HBsAg^[15]. In some regions with high endemicity (such as China), about 1/3 or more of cirrhotic patients with negative HBsAg may harbor HBV infection^[15].

The rate of HBV infection in Iran is unknown but it is the most frequent cause of chronic hepatitis, cirrhosis and hepatocellular carcinoma. It appears that Iran is in a region with intermediate or even low endemicity and the rate of positivity of HBsAg and HBcAb in the general population is 1.8%-5% and over 35% respectively^[16].

The goal of the present study was to determine whether a negative test for HBsAg in the serum is sufficient to rule out the possibility of chronic HBV hepatitis in Iranian patients with chronic liver disease and to assess the possible role of "a" determinant mutations in Iranian patients with chronic HBV hepatitis. We conducted a prospective study to determine the presence of HBV-DNA in serum of patients with chronic hepatitis and negative HBsAg.

MATERIALS AND METHODS

Patients

After completion of a questionnaire and physical examination, serum samples were collected from 104 adult (> 16 years old) patients (69 males and 35 females) with

chronic (6 mo or more) high alanine amino-transferase (ALT) and negative HBsAg who were referred for liver biopsy to endoscopy wards of Faghihi and Nemzee Hospitals, Shiraz University of Medical Sciences, Shiraz, Fars province, south of Iran, between February 1997 to December 2001.

Diagnosis of chronic hepatitis was based on a minimum of 6 mo or more persistent elevation of ALT (more than 60 or 1.5 times of upper limit of normal value) and grade of necro-inflammation of more than 3/18 of Ishak (modified Knodell) classification in liver histology. All active alcohol users (drinking of more than 20 g alcohol per day), patients with biliary obstruction, cardiac failure, active cancer or chronic infection (such as tuberculosis) and cirrhosis (shrunken liver in ultrasonography and regenerative nodules in histology and one of these features: history compatible with grade 2 of hepatic encephalopathy, endoscopically confirmed esophageal or fundal varices, ascites, hypersplenism), positive HBsAg, positive anti-smooth muscle antibody, positive anti-nuclear antibody, positive anti-mitochondrial antibody, low titers of alpha-one-anti-trypsin or ceruloplasmin, abnormal iron or copper levels, predominant steatohepatitis, biliary duct injury or congestion (Budd-Chiari syndrome) in liver histology were excluded from the study.

Demographic, biochemical and serological data, histological findings and results of PCR of our patients are shown in Table 1.

Serological assays and PCR

All serological tests including HBsAg, IgG-anti HBcAb, second generation of anti-hepatitis C virus antibody (HCVAb), hepatitis B e antigen (HBeAg) and IgG-antibody to hepatitis B e antigen (HBeAb) were performed using ELISA based on manufacturers' instructions.

PCR-assay

In order to prevent any contamination, we strictly adhered to the standard guideline of PCR procedure. Fifty microliters of serum were diluted 1:5 with 250 μ L NaOH (50 mmol/L) for DNA denaturation and RNA inactivation. Samples were then heated at 95°C for 20 min to denature proteins. Forty microliters of Tris 1 mmol/L (pH = 7.3) was then added and centrifuged at 14000 r/min for 7 min. Forty microliters of phenols-chloroform (1:1) were added and centrifuged at 14000 r/min for 7 min. Ten microliters of sodium acetate (3 mol/L) and 300 μ L absolute ethanol were added to each tube. After incubation at -70°C for 40-60 min, tubes were centrifuged at 14000 r/min for 10 min. Supernatant was removed and 50-100 μ L of TE buffer (pH = 7.8) was added to the pellets. PCR-amplification was performed using published oligonucleotide primers^[17] selected from highly conserved HBV surface gene, whereby primer 1 and primer 2 flank 597 base-pair fragment.

Five microliters of sample were added to 45 μ L of reaction mixture (2.5 U Tag polymerase, 22.5 μ mol/L of each primer, 200 μ mol/L of each deoxynucleotide triphosphate, 5 μ L of reaction buffer [50 mmol/L KCl, 10 mmol/L Tris-HCl pH = 8.3 and 1.5 mmol/L MgCl₂]). Samples were denatured for 5 min at 94°C and then subjected to 40 cycles of 1 min at 58°C, 1 min at 72°C and 1 min at 94°C

Table 1 Demographic, biochemical and serological data and histological findings

Variable	Result
Sex (M/F)	69/35
Age (yr)	Mean: 34.8 \pm 10.3, Min: 17, Max: 71
Mean AST nkat/L (\pm SD) ¹	2312.1 (\pm 373.4)
Mean ALT nkat/L (\pm SD) ¹	2525.5 (\pm 430.1)
Mean ALK Ph ² nkat/L (\pm SD) ³	6656.3 (\pm 943.5)
HCV Ab (+/-)	68/36
Serum HBV-DNA (+/-)	2/102
Mean grade of necro-inflammation (of 18) (\pm SD)	8.2 (\pm 3.4)
Mean stage of fibrosis (of 6) (\pm SD)	3.1 (\pm 1.7)

¹Normal < 667 nkat/L, ²Alk Ph means alkaline phosphatase, ³Normal: 1084-5251 nkat/L.

in an Eppendorf thermal cycler (Master cycler 5330).

Ten microliters of reaction product was electrophoresed in a 18 g/L agarose gel made in Tris-acetate-EDTA (TAE) buffer (pH = 8-8.5) and visualized by ultra-violet illumination after ethidium bromide (10 mg/L) staining. Positive and negative controls were also treated as samples.

PCR amplification was also performed using HBV-PCR detection kit (Cinna Gen Inc., Teheran, Iran). According to manufacturer's instruction, 5 μ L of extracted DNA was added to 20 μ L of a PCR mixture, which contained primers amplifying 353 bp of the region of the core gene. Samples were denatured for 2 min at 94°C and then subjected to 40 cycles of 93°C for 40 min, 62°C for 40 min, 72°C for 40 min and finally 72°C for 10 min. The sensitivity of PCR assay was 150×10^3 copies/mL.

RESULTS

Only two patients had HBV DNA in their sera. Demographic, biochemical data, serological data, and histological findings of these patients are shown in Table 2. Both patients had major thalassemia and were on regular blood transfusion for many years.

DISCUSSION

There is much controversy and suspicion about the possible role of hepatitis B infection in pathogenesis of cryptogenic chronic parenchymal liver diseases especially in areas with intermediate or high infection rates of hepatitis B. Although the exact mechanism for active viral infection with undetectable HBsAg in the serum is not clear, both mutations in S region of the viral genome as well as low-titer infections (including possible burned-out infections) are proposed as possible explanations. It is however obvious with either of these two mechanisms being true, there should be detectable viral genome in serum of a majority of such patients using sensitive amplification techniques.

In this study, only less than 2% of our patients had HBV-DNA in their sera and it appears that HBV infection had a minimal role in causing chronic hepatitis in HBsAg negative Iranian patients with chronic hepatitis. Because of lack of a control group of normal individuals to which

Table 2 Demographic, biochemical, serological and histological data of patients with positive HBV-DNA

Variable	Patient No. 18	Patient No. 47
Age (yr)	21	27
Sex	M	F
AST nkat/L (\pm SD) ¹	1617	2917.3
ALT nkat/L (\pm SD) ¹	1667	2334
Alk Ph ² nkat/L (\pm SD) ³	2133.8	8568.4
Grade of necro-inflammation (of 18)	4	18
Stage of fibrosis (of 6)	3	5
HBsAb	-	+
HBcAb	+	-
HBsAg	+	+
HBcAb	-	-
HCV-Ab	+	+

¹Normal < 667 nkat/L; ²Alk Ph means alkaline phosphatase; ³Normal: 1084-5251 nkat/L.

the incidence of positive HBV DNA in serum can be compared, the possible role of occult HBV infection in etiology of cryptogenic liver disease in this country cannot be totally excluded, nevertheless, it can be certainly stated that this role if, exist at all will be of minimal, negligible impact.

This data is the same as that found by western researchers but in contradiction with findings in high incidence areas of the world^[15]. The possible explanation for these different results may lie in the different incidences as well as different genotypes of hepatitis B prevalence in these areas.

It should be further emphasized that two patients with positive HBV DNA in this study were both known cases of major thalassemia with a history of multiple transfusions and also had concomitant HCV infection. It is therefore can be concluded that the incidence of occult HBV infection in those with no history of high risk conditions for blood borne infections will be much lower.

Based on the findings of this study, we conclude that there is no need for HBV DNA PCR in routine work up for the chronic liver disease patients with negative HBsAg in Iran.

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Differentiation of rat bone marrow stem cells in liver after partial hepatectomy

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Abstract

AIM: To investigate the differentiation of rat bone marrow stem cells in liver after partial hepatectomy.

METHODS: Bone marrow cells were collected from the tibia of rat with partial hepatectomy, the medial and left hepatic lobes were excised. The bone marrow stem cells (Thy⁺CD3⁺CD45RA⁻ cells) were enriched from the bone marrow cells by depleting red cells and fluorescence-activated cell sorting. The sorted bone marrow stem cells were labeled by PKH26-GL *in vitro* and autotransplanted by portal vein injection. After 2 wk, the transplanted bone marrow stem cells in liver were examined by the immunohistochemistry of albumin (hepatocyte-specific marker).

RESULTS: The bone marrow stem cells (Thy⁺CD3⁺CD45RA⁻ cells) accounted for 2.8% of bone marrow cells without red cells. The labeling rate of 10 μ M PKH26-GL on sorted bone marrow stem cells was about 95%. There were sporadic PKH26-GL-labeled cells among hepatocytes in liver tissue section, and some of the cells expressed albumin.

CONCLUSION: Rat bone marrow stem cells can differentiate into hepatocytes in regenerative environment and may participate in liver regeneration after partial hepatectomy.

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Key words: Bone marrow stem cells; Liver regeneration; Differentiation

INTRODUCTION

Recent studies indicate that mice and human bone marrow cells can differentiate into hepatocytes in normal liver and rat bone marrow cells can turn into hepatocytes in severely damaged liver with the suppression of hepatocyte proliferation^[1-6]. However, whether rat bone marrow stem cells can differentiate into hepatocytes in liver after partial hepatectomy remains unclear. The aim of this study was to investigate the differentiation of rat bone marrow cells in regenerative environment after partial hepatectomy.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats weight 170-190 g were obtained from the Animal Center of Peking University People's Hospital. They were allowed to have free access to standard laboratory chow and kept in a 12 h light/dark cycle.

Materials

Erythrolysin, phycoerythrin-conjugated mouse anti-rat CD45RA and phycoerythrin-conjugated mouse anti-rat CD3 were purchased from Becton Dickinson Inc. Fluorescein isothiocyanate-conjugated mouse anti-rat Thy-1.1 was obtained from Pharmingen Inc. PKH26-GL was purchased from Sigma Inc. Mouse anti- albumin antibody was obtained from Dako Inc. FITC-labeled anti- mouse IgG was supplied by Zhongshan Inc. RPMI 1640 was from Gibco Inc.

Partial hepatectomy rat model

The rats were anesthetized with intraperitoneal injection of sodium pentobarbital (35 mg/kg body weight). Local skin was sterilized by routine method. Partial hepatectomy rat model was established by resecting medial and left liver lobes.

Bone marrow stem cell enrichment

Under general anesthesia, bone marrow was aspirated

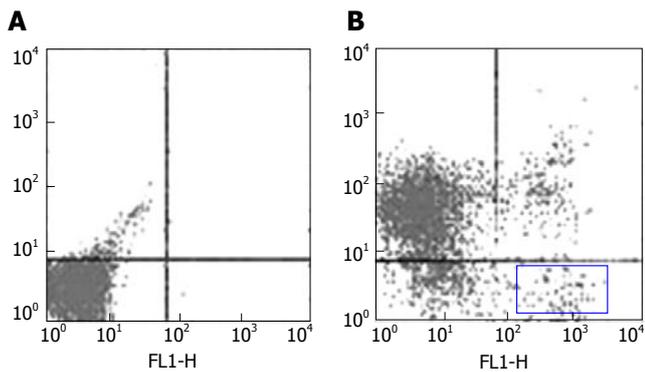


Figure 1 Bone marrow stem cells sorted by flow-cytometry. **A:** Control; **B:** Percentage of Thy⁺CD3⁺CD45RA⁻ cells in bone marrow cells without erythrocytes (about 2.8%).

from tibia with a syringe containing 1 mL heparin with an 18-gauge needle. The marrow cells were transferred to a sterile tube and mixed with 10 mL culture medium (RPM1640 supplemented with 10% fetal bovine serum, 100 μ /mL penicillin G and 100 μ g/mL streptomycin). Red blood cells in bone marrow were depleted by erythrolysin. After washed three times with phosphate-buffered saline (PBS), the cells were incubated at 4°C for 30 min with fluorescein isothiocyanate-conjugated anti-Thy, phycoerythrin-conjugated anti-CD3 and phycoerythrin-conjugated anti-CD45RA. The cells were washed three times and resuspended in medium. Labeled cells were analyzed and separated with FACS-vantage (Becton Dickinson, San Jose, CA). Gating was based on Thy-positive, CD3-negative and CD45RA-negative.

Bone marrow stem cell staining with PKH26-GL

The sorted Thy⁺CD3⁺CD45RA⁻ cells were labeled by 10 μ M PKH26-GL. Labeling conditions employed were essentially as described by the manufacturer. Briefly, cells were suspended in Diluent C at a density of 2×10^6 cells/mL and mixed with an equal volume of the PKH26-GL dye in Diluent C to give a final dye concentration of 10 μ mol/L. Labeling was carried out at room temperature for 3 min. The labeling reaction was terminated by the addition of an equal volume of fetal bovine serum. Labeled cells were diluted with culture medium containing serum and washed three times.

Bone marrow stem Cell transplantation

The PKH26-GL-labeled bone marrow stem cells were suspended in 300 μ L of RPM1640 without serum at a concentration of 1×10^6 cells and autotransplanted by portal vein injection.

Immunocytochemistry

The liver tissue was obtained at two weeks after partial hepatectomy. Frozen sections were made and incubated overnight at 4°C in 50 μ L anti-albumin antibody (1:50), then washed three times in PBS and incubated for 1 h at 37°C in 50 μ L FITC-labeled second antibody. After washed three times with PBS, the slides were occluded by glycerin buffer and observed under confocal laser scanning

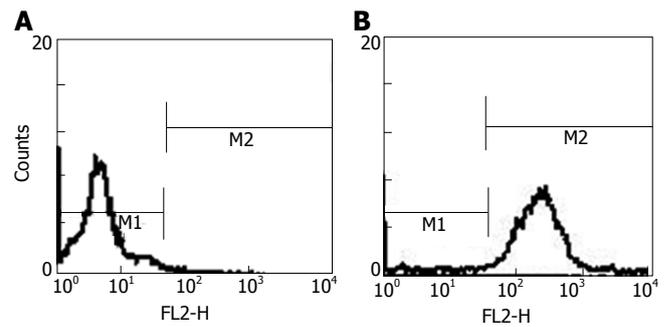


Figure 2 PKH26-GL labeling of Thy⁺CD3⁺CD45RA⁻ cells. **A:** Control; **B:** Percentage of PKH26-GL-labeled cells in Thy⁺CD3⁺CD45RA⁻ cells. (M1: unlabeled cells; M2: labeled cells).

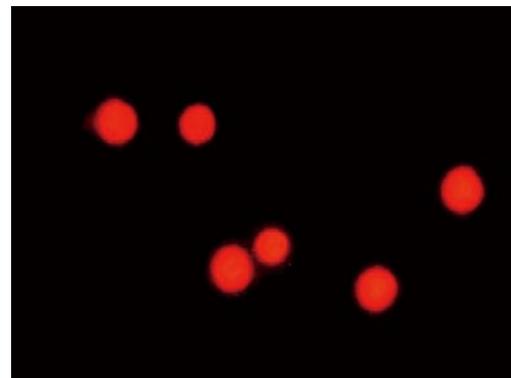


Figure 3 Red Thy⁺CD3⁺CD45RA⁻ cells with PKH26-GL under fluorescence microscope.

microscope.

RESULTS

Bone marrow stem cell enrichment

After erythrocytes were depleted in tibia bone marrow cells from rats after partial hepatectomy. Bone marrow stem cells were enriched by sorting the Thy⁺CD3⁺CD45RA⁻ cells. The sorted cells accounted for about 2.8 % (Figure 1).

Enriched bone marrow stem cell staining with PKH26-GL

The Thy⁺CD3⁺CD45RA⁻ cells were labeled with 10 μ M PKH26-GL. The percentage of labeled cells was about 95% (Figure 2). The labeled Thy⁺CD3⁺CD45RA⁻ cells were red under fluorescence microscope (Figure 3). PKH26-GL had no obvious effect on bone marrow cell viability. Trypan blue staining assay showed that the cell viability of PKH26-GL-labeled bone marrow cells was higher than 85%.

Immunohistochemistry staining for albumin

PKH26-GL could emit red fluorescence at the wavelength of 567 nm, while FITC could emit green fluorescence at the wavelength of 494 nm. We tested liver tissue sections under confocal laser scanning microscope at the two wavelengths respectively. There were sporadic cells with red fluorescence among hepatocytes in liver sections (Figure 4A), suggesting that PKH26-GL-labeled bone marrow

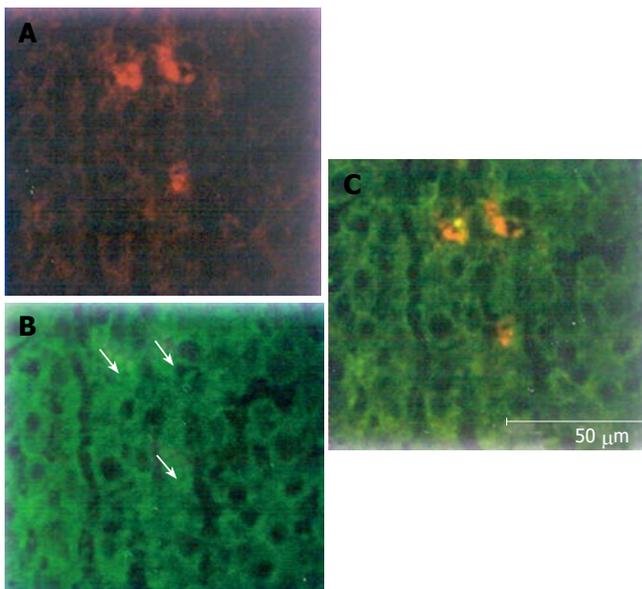


Figure 4 Red sporadic PKH26-GL-labeled cells (A), green hepatocytes (B), and yellow PKH26-GL-labeled cells expressing albumin (C).

stem cells could migrate into liver. Hepatocytes showing green fluorescence suggested that hepatocytes expressed albumin (Figure 4B). Yellow was mixed with red and green. Yellow cells derived from PKH26-GL labeled *in vitro* bone marrow stem cells expressed albumin (Figure 4C). The findings indicated that bone marrow stem cells could differentiate into hepatocytes in regenerative hepatic environment after partial hepatectomy.

DISCUSSION

Seventy percent hepatectomy rat model is a classical animal model to study liver regeneration. The remaining hepatic lobes of the model rat can restore the liver mass at 2 wk after partial hepatectomy. It has been considered that the regeneration process is due to the proliferation of residual mature hepatocytes. Recently, important developments have been achieved in the field of stem cell study. Petersen *et al.*^[3] used 3 separate approaches to follow transplanted bone marrow cells and found an extrahepatic source for the liver cells. In a careful histological analysis of irradiated female mice that received male donor cells, the Y chromosome could be detected in some hepatocytes 2 to 6 mo after transplantation in the absence of any intentional liver injury^[7]. In human female recipients of male bone marrow, some hepatocytes contain the Y chromosome^[8]. These findings suggest that there is a linkage between bone marrow cells and liver. It is hypothesized that, similar to other organ systems, the liver has 3 levels of cells: “mature” hepatocyte, original tissue-determined stem cells represented in the adult organ by cells in the terminal bile ductules (canals of Hering), and a multipotent stem cells in the liver derived from circulating bone marrow stem cells^[9]. However, whether circulating bone marrow stem cells can differentiate into hepatocytes in regenerative liver after partial hepatectomy remains unclear.

PKH26-GL shows little or no toxicity except for some

phototoxicity following prolonged exposure of PKH26-GL labeled cells to excitation light and can be used to track lymphocyte migration for weeks to months^[10,11]. It is the dye of choice for cell migration and proliferation studies. PKH26-GL has been used to *in situ* label mouse spleen cells and peripheral blood neutrophils, and is particularly effective in monitoring the *in vivo* homing and proliferation of haemopoietic stem cells^[12-14]. Our study showed that 10 $\mu\text{mol/L}$ PKH26-GL could effectively label rat bone marrow cells and has no obvious effect on cell viability. PKH26-GL-labeled bone marrow cells were autotransplanted in rats during partial hepatectomy. After 2 wk, there were PKH26-GL-labeled cells in liver, and the cells expressed hepatocyte-specific marker albumin. The result showed that bone marrow cells could differentiate into hepatocytes in regenerative hepatic environment. Bone marrow cells consist of white cells, erythrocyte, a few stem cells, *etc.* Only stem cells could differentiate into other type cells. As a result, the experiment indicated that circulating bone marrow stem cells could differentiate into hepatocytes in liver after partial hepatectomy and bone marrow stem cells might participate in hepatic regeneration. Fujii^[15] examined the differentiation of mice bone marrow cells in liver regeneration after partial hepatectomy and found that bone marrow cells participate in liver sinusoid. They believe the bone marrow cells participation in liver regeneration after hepatectomy, where the majorities were committed to sinusoidal endothelial cells probably through endothelial progenitor cell mobilization.

It has been accepted that the hepatocyte regeneration process after partial hepatectomy is associated with the proliferation of remaining hepatocytes. We hold that the mechanism of hepatocyte regeneration after partial hepatectomy includes at least two ways. That is, the remaining mature hepatocytes proliferate and circulating bone marrow stem cells migrate into residual liver and differentiate into hepatocytes. With the development and application of stem cell technique, it is possible to obtain stem cells from bone marrow or blood and to make the stem cells proliferate in a great deal. Stem cell autotransplantation may become a new method to promote liver regeneration.

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Preoperative risk factor analysis in orthotopic liver transplantation with pretransplant artificial liver support therapy

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Abstract

AIM: To assess the value of pre-transplant artificial liver support in reducing the pre-operative risk factors relating to early mortality after orthotopic liver transplantation (OLT).

METHODS: Fifty adult patients with various stages and various etiologies undergoing OLT procedures were treated with molecular adsorbent recycling system (MARS) as preoperative liver support therapy. The study included two parts, the first one is to evaluate the medical effectiveness of single MARS treatment with some clinical and laboratory parameters, which were supposed to be the therapeutical pre-transplant risk factors, the second part is to study the patients undergoing OLT using the regression analysis on preoperative risk factors relating to early mortality (30 d) after OLT.

RESULTS: In the 50 patients, the statistically significant improvement in the biochemical parameters was observed (pre-treatment and post-treatment). Eight patients avoided the scheduled Ltx due to significant relief of clinical condition or recovery of failing liver function, 8 patients died, 34 patients were successfully bridged to Ltx, the immediate outcome of this 34 patients within 30d observation was: 28 kept alive and 6 patients died.

CONCLUSION: Pre-operative SOFA, level of creatinine, INR, TNF- α , IL-10 are the main preoperative risk factors that cause early death after operation, MARS treatment before transplantation can relieve these factors significantly.

Key words: Liver transplantation; Artificial liver; Sequential Organ Failure Assessment; Risk factors analysis

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INTRODUCTION

Orthotopic liver transplantation (OLT) is an accepted mode of therapy for selected patients with advanced liver diseases, however, the early mortality after OLT remains relatively high due to the poor selection of candidates with serious conditions. The immediate outcome of OLT is dependent on many factors including pre-transplantation conditions such as renal function and some other organ functions, which indicate that the better preparation for stability or improvement of the previous OLT support, the better immediate outcome for the OLT. It is now widely accepted that the artificial liver support can be of the optional therapy to stabilize patients with liver failure and to gain time for recovery or re-compensation of the liver or to get a suitable donor available^[1]. Although the hybrid biologic method seems theoretically rational, criticism of and arguments against the concept in fear of rejection, transmission of porcine viruses and inducing release of cytokines such as TNF- α and IL-10 probably hinder these methods from gaining a prominent role in future liver support strategies^[2]. A new innovated blood purification system based on hemodialysis named molecular adsorbent recycling system (MARS) has been applied to a wide variety of hepatic failure patients and represents positive effects, the method was shown to be efficient in removing both hydrosoluble substances and strongly albumin-bound substances. The remarkable removal of strongly albumin-bound substances and kinds of metabolic toxins during MARS could therefore reduce the toxic effects and higher concentrations of these compounds exert on liver and kidney, thus could contribute to improvement in encephalopathy, renal and hepatic function and probably survival^[3,4].

The aim of this study is to assess the value of pre-transplant artificial liver support in reducing the preoperative risk factors relating to early mortality after OLT.

MATERIALS AND METHODS

Patients and design

Fifty adult patients undergoing OLT procedures with MARS preoperative liver support therapy in our transplantation institute from September 2002 to June 2003 were studied. The study population consisted of 28 men and 22 women with a mean age of 52.24 years (range 46-68). The indications for liver transplantation were post-hepatic cirrhosis ($n = 11$), fulminant hepatic failure ($n = 6$), alcoholic cirrhosis ($n = 5$), primary liver cancer ($n = 7$), primary biliary cirrhosis ($n = 12$), Wilsons disease ($n = 6$) and post-operative rejection ($n = 3$).

The study contains two parts, the first one is to evaluate the medical effectiveness of single MARS therapy with some clinical and laboratory parameters which were supposed to be the therapeutical pre-transplantation risk factors, the second part is to study the patients undergoing OLT using the regression analysis in preoperative risk factors relating to early mortality (30 d) after OLT.

Our trial was approved by the Ethical Committee of Human Experimentation in China, and was in accordance with the Helsinki Declaration of 1975. All procedures were performed after informed and written consent had been obtained from the patients or their kins.

Treatment and monitoring of clinical and laboratory parameters

Initially, patients were given conventional medical therapy and intensive monitoring or supportive treatment. They were evaluated biochemically and clinically with the prognostic scoring systems including Sequential Organ Failure Assessment (SOFA) score, Glasgow coma score and Hepatic encephalopathy (HE) grade before and after the treatment, no sedatives were administered during treatment, the laboratory variables as Tumor Necrosis Factor α (TNF- α), and Interleukin-10 (IL-10) levels were detected with the flow cytometer (FACSCalibur, Becton, Dickinson and Company, USA).

Those patients who were bridged to OLT were divided into two groups: survival group (survival time ≥ 30 d) and death group (survival time ≤ 30 d). There were different possible risk factors relating to early mortality after liver transplant including pre-operative, inter-operative and post-operative ones. Pre-operative information consisted of age, SOFA, mean artery pressure (MAP), serum Aspartate Transaminase (AST), serum albumin, International Normalized Ratio (INR), serum creatinine level (Cr), serum total bilirubin level, bile acid, serum ammonia, TNF- α , and IL-10.

The extracorporeal liver support system (MARS)

The MARS system (MARS monitor, Teraklin AG, Rostock, Germany) was used with a continuous veno-venous hemofiltration (CVVH) machine (GAMBRO AK 200), The MARS treatment was performed according to the recently published guidelines^[3], a vascular access via a double-lumen jugular central vein line was created, low molecular weight heparine (LMWH) was used for blood anticoagulation for the maintenance of the extracorporeal circuit. The blood

Table 1 Comparison of clinical and biochemical data pre-and post MARS treatment

Parameter	Pre-treatment	Post-treatment	P value
Prognostic scores			
SOFA score (points)	9.72 \pm 1.89	6.98 \pm 2.34	< 0.01
Glasgow coma score (points)	7.23 \pm 1.21	13.34 \pm 2.33	< 0.01
Clinical and biochemical parameters			
MAP (mmHg)	70.5 \pm 12.1	85.1 \pm 10.4	< 0.001
TNF- α (pg/mL)	2.83 \pm 1.7	1.80 \pm 1.39	< 0.01
IL-10 (pg/mL)	7.80 \pm 6.0	4.5 \pm 4.5	< 0.05
ALT	295.7 \pm 1125.4	237.14 \pm 91.82	< 0.036
TBIL (μ mol/L)	341.16 \pm 94.93	232.74 \pm 169.29	< 0.04
TBA (mmol/L)	162 \pm 104	73 \pm 51	< 0.001
Albumin (g/L)	35.5 \pm 35.7	35.7 \pm 6.8	> 0.05
INR	4.66 \pm 3.35	3.98 \pm 3.16	> 0.05
Cr (μ mol/L)	128.67 \pm 87.73	33.21 \pm 22.26	< 0.045
Ammonia (mmol/L)	151.31 \pm 88.99	28.28 \pm 20.19	< 0.028

flow from the dialysis machine and the albumin dialysate circuit flow were equal at a rate of 80-120 mL/min, while the dialysate flow was set accordingly. The time length of each therapeutic session was 6-8 h.

Statistical analysis

Experiment data were presented as mean \pm SD, independent 2-tailed *t* tests were used to determine whether there were significant differences between the survival and dead group of OLT patients. Pearson's Chi-square statistics were used to test differences in all frequencies. Data with significant difference were entered into a stepwise logistic regression analysis.

RESULTS

In the 50 patients, 80 MARS treatments were performed. Patients who underwent the intended MARS treatment were all remarkably stable without any adverse events or complications except slight chill. All patients showed positive response to the therapy, with respect to remarkable release of severe meteorism, active diet, and significant improvement of liver and kidney functions. SOFA, GSH, MAP, TNF- α and IL-10 were improved significantly, the total bilirubin (TBIL), total bile acid (TBA), ammonia and creatinine (Cr) were shown decreased markedly, however, no difference was found in the markers of alanine-aminotransferase (ALT), albumin and INR before and after the MARS treatments (Table 1). Eight patients avoided the scheduled Ltx due to significant relief of clinical condition or recovery of failing liver function, 8 patients died, 34 patients were successfully bridged to Ltx, the immediate outcome of these 34 patients within 30d observation was: 28 kept alive and 6 patients died.

Comparison between the survival group and the death group showed significant differences in terms of age, SOFA, GSH, MAP, TNF- α , IL-10, INR and serum creatinine level ($P < 0.05$, Table 2). The stepwise logistic regression was used to create the best statistical model

Table 2 Risk factors relating to early mortality after liver transplantation

Group	n	Age ^a	SOFA ^b	GSH ^a	MAP ^a	TNF- α ^a	IL-10 ^a	INR ^a	Creatinine ^b
Survival	28	34.34 \pm 2.45	8.18 \pm 1.23	12.45 \pm 2.26	83.7 \pm 10.01	1.99 \pm 1.4	5.5 \pm 2.0	3.34 \pm 1.89	45.45 \pm 12.12
Death	6	45.56 \pm 2.36	9.88 \pm 1.09	9.88 \pm 2.43	75.0 \pm 12.46	2.34 \pm 1.05	6.2 \pm 2.9	4.77 \pm 2.07	110.34 \pm 23.65

^aP < 0.05, ^bP < 0.01.

Table 3 Stepwise logistic regression analysis

Variables	B	SE	Wald	Df	Sig	R
SOFA	2.638	0.879	5.879	1	0.018	0.224
Creatinine	5.822	1.351	8.345	1	0.002	0.315
TNF-A- α	2.131	0.775	5.664	1	0.019	0.192
IL-10	2.014	0.766	4.784	1	0.025	0.188
INR	3.455	1.335	8.025	1	0.002	0.309

relating to early mortality after transplantation. The factors that had significant independent associations with early mortality after the stepwise procedure were serum creatinine level, INR, SOFA, TNF- α and IL-10, with regression coefficients of 0.315, 0.309, 0.224, 0.192 and 0.188, respectively (Table 3).

DISCUSSION

OLT is a treatment for end-stage liver disease. Early post-operative mortality was below 10% in the world. The mortality remains high in our country. The serious preoperative condition of recipient and the late timing for operation may account for this result. The serious preoperative conditions are due to the poor detoxification ability of the liver, the accumulation of large amounts of toxic substances including hydro-soluble and lipid soluble and various metabolic products, which causing further damage to the heart, brain, kidney and vessels leading to MODS. The accumulation will increase the mortality of OLT^[5]. So, in order to increase the successful rate of OLT, the toxic substances shall be removed as much as possible before operation. The removal of the toxins will help block the vicious cycle and benefit the organ function.

The albumin dialysis MARS is currently the novel cell-free liver support system which enables the selective removal of water-soluble and albumin-bound substances, in which human serum albumin serves as a shuttle based on facilitated diffusion between a blood-sided dialysis membrane on one side and a remove set of sorbent columns and a conventional dialysis unit on the other^[2,3]. MARS has avoided the drawbacks of conventional and bio-artificial liver. It combines the functions of molecular adsorption, hemo-filtration and hemo-dialysis, and can remove the albumin combined toxins quickly and selectively. The hemo-filtration can do nothing to the albumin combined toxins. The hemo-perfusion, whose adsorbents such as carbon or resin will contact with blood directly, cause fibrolysis or activation of complements and systemic

inflammation due to the bio-incompatibility, and also, cause the loss of hepatic cytokines and a large amount of hormones such as T3, T4 and insulin. The plasma exchange has very weak ability to remove toxins and causes loss of useful substances. Attempts to develop an artificial liver were started in the 1950s, today both mechanical devices and hybrid biologic-mechanical devices such as cell-free detoxification methods, whole organ perfusion, hepatocytes transplantation and bioreactor approaches using hepatocytes, however, are with inherent defectiveness and limitations respectively. Although the hybrid biologic methods seem theoretically rational, criticism of and arguments against the concept in fear of rejection, transmission of porcine viruses and inducing release of cytokines as TNF- α and IL-6 probably hinder these methods from gaining a prominent role in future liver support strategies. Many randomized, controlled trials were performed, all reported with statistically significant increases in survival in the MARS group compared with standard care, indications for treatment were hepato-renal syndrome, decompensated chronic disease with intrahepatic cholestasis, post-operative liver failure after heart surgery and cirrhosis with superimposed acute liver injury. The laboratory data of pre- and post-treatment in the 80 sessions on the 50 patients has proved the conclusion of some other researchers. The conclusion is that MARS can remove various toxins and cytokines caused by liver failure, decrease SOFA score and improve multi-organ function.

In this study, we are trying to explore the effects of MARS treatment in post-operative early survival rate. Aiming at this purpose, we analyzed multiple factors which may be related to the post-operative mortality rate. It is different with the bygones. We only analyzed some pre-operative factors which are operable, measurable and curable although the factors are related to the post-operative mortality comprise of not only post-operative factors, but also intra- and post-operative ones such as ischemia time, ahepatic time, bleeding volume and rejection or various complications. Some factors, though very important, can't be controlled or cured, which are not drawn into our study, such as the pathology, former related operation or former transplantation history, ABO blood group, *etc.* The early mortality rate is 17% in the 34 patients in this study. We adopts Logistic regression analysis. The results show that preoperative SOFA, levels of creatinine, INR, TNF- α and IL-10 are the main preoperative risk factors causing early death after operation.

Renal dysfunction is a common dangerous complication in patients with end-stage liver disease. It results from acute tubular necrosis and caused by

hepatorenal syndrome. Literature shows a significant decrease of 43% in glomerular filtration rate (GFR) during transplantation in patients with normal renal function. Both high pre-operative serum creatinine level and inter-operative veno-venous bypass can lead to renal hemodynamic instability during operation. The post-operative nephrotoxic immuno-suppressants can also contribute to the irreversible renal insufficiency, leading to renal failure after liver transplantation. In our study, 2 of 10 patients died of renal failure, who were all along with high preoperative serum creatinine level and hepatorenal syndrome before transplantation. A significant difference found between the survival group and the death group was shown in serum creatinine level ($P < 0.001$), with a mean of 45.45 ± 12.12 and 110 ± 23.65 , respectively. The stepwise logistic regression analysis also showed a statistical independent association with early mortality after liver transplantation, with a regression co-efficient of 0.315. It is also a main risk factor which predicts early mortality after OLT. It is very important to improve renal malfunction before transplantation. If there are indications for hemodialysis, it should be performed without delay, trying to relieve the renal function before operation^[6]. The MARS is a recommended strategy for this kind of case. Because, not only can MARS lower the level of blood creatinine, but also raise mean artery pressure (MAP) and systemic vascular resistance (SVR), for it can lower the blood level of NO which is a vessel dilator to the liver failure patients^[7].

SOFA is currently a popular system applied in ICU for the assessment of multi organ function. SOFA covers the function of multi-system completely including respiratory, hemostatics, hepatic, circulatory, and of brain and kidney. Comparing with the widely used Acute Physiology and Chronic Health Evaluation (APACHE), SOFA has the advantage of daily sequential assessment, and is better than APACHE which is not specific enough to liver function and also better than Child-Turcotte-Pugh (CTP) system which is too specific to liver function^[8]. In this study, the SOFA score is 9.88 ± 1.09 , which is significantly higher than 8.18 ± 1.23 in the survival group ($P < 0.001$). Logistic regression analysis showed that SOFA is related to early mortality in certain extent, its regression coefficient is 0.224. SOFA is one of the main risk factors. It can be regarded as a reference index in choosing operation time.

Though the association between pre- and post-operative level of TNF- α and IL-10 is not so significant as the other risk factors, it still deserves much consideration. A lot of studies show that the systemic inflammatory responses syndrome (SIRS) caused by TNF- α and IL-10 is an important tache to the aggravation of liver failure leading to MODS. Not only does SIRS play an important role in the development of liver failure, but also makes a hormone-like effect, causing hypotension, lung injury, brain edema *etc.* So SIRS may be the common body fluid causing multi-organ injury. If we can remove these factors, theoretically, the MODS caused by the strike of the operation can be relieved, which is beneficial to increasing the success rate of operation. In this study, three patients died of MOF (multi-organ failure). The study showed that

the values of preoperative parameters were lower in the survival group, which may be due to being removed of the cytokine and being blocked of injury to organs by SIRS. The relief of oxygenation of the body will also do benefit to preventing MODS^[9]. Recent studies show that the TNF- α and IL-10 play important roles in mediating the inflammation and immune response caused by rejection. The variability of the cytokine initiator is a risk factor which is related to rejection. Whether the removal of preoperative TNF- α and IL-10 will be of benefit to relieve post-operative rejection is awaits further studies^[10].

In this study, we find that MARS can improve multi-organ function both before and after operation. The improvement is presented as the decreased SOFA score. This is in agreement with some reports in the world. The mechanism may be that MARS has selectively removed a large amount of albumin bound toxins and hydrosoluble toxins including cytokines such as TNF- α , IL-10 and endo-toxin, and repressed or relieved the pathological progress, and also improved the body blood circulation, organ blood dynamics and tissue oxygenation, benefiting the intervention on MODS. Regarding the risk factors, this preoperative MARS treatment will ensure a low post-operative MODS rate and increase the success rate of operation.

Furthermore, INR is an important risk factor related to post-operative early mortality. High preoperative INR is related to disfunction of blood clotting, leading to interoperative bleeding. Large amounts of blood transfusion may develop hemodynamic unsteadiness or even DIC. The long-term hypotension will aggravate the renal failure which has already existed before operation. INR acts as the medium of blood clotting mechanism. Abnormal INR accompanied with previous upper abdominal surgery, or extensive abdominal adhesions, portal hypertension and hypersplenism may develop severe bleeding. It is reported that intra-operative bleeding quantity is related to early mortality after liver transplantation^[11,12]. In this study, INR is not significantly improved by single MARS treatment, possibly because single MARS treatment is not enough to significantly improve of the synthesizing ability of the liver. But it will be improved after sequential treatment. Unlike the other artificial liver treatment, MARS is featured by high bio-compatibility and stable hemo-dynamics. It will not do harm to or initiate the disorder of blood clotting. MARS is very useful in stabilizing INR which is related to the prognosis.

Some other factors such as Glasgow Score (GSH) and mean artery pressure (MAP) showed associations with early mortality after liver transplantation, but the significance is not as high as SOFA and creatinine.

CONCLUSION

After studying the laboratory data of pre- and post-treatment in the 80 sessions on the 50 patients, we think that MARS can be used in transition to OLT. Preoperative SOFA, level of creatinine, INR, TNF- α and IL-10 are the main preoperative risk factors that cause early death after

operation. MARS treatment before transplant operation can relieve these factors significantly.

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RAPID COMMUNICATION

Comparison of therapeutic effectiveness of combined interventional therapy for 1126 cases of primary liver cancer

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effect of TACE and RFA is better than that of TACE, and the effect of TAI is minimal.

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Key words: Hepatocellular carcinoma; Transcatheter arterial chemoembolization; Combined interventional therapy; Survival analysis

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Abstract

AIM: To verify the effect of combined interventional therapy for hepatocellular carcinoma (HCC).

METHODS: The clinical data of 1126 HCC patients who received combined interventional therapy for transcatheter arterial chemoembolization (TACE) before or after hepatectomy, TACE and radio-frequency ablation (RFA), Chinese medicine treatment and biotherapy after TACE or transcatheter arterial infusion (TAI), were reviewed according to the results of their liver function, alpha-fetoprotein, image data, color-ultrasonography finding and survival rate.

RESULTS: A total of 874 patients were followed up for a period of 2 to 63 mo. The overall 1-, 3- and 5-year survival rates were 67.8%, 28.7% and 18.8% respectively. The 1- 3- and 5- year survival rates of patients who received TACE were 74.7%, 41.4%, 36.9% before hepatectomy and 78.9%, 40.4%, 37.5% after hepatectomy. The effective rate (PR + NC) after TACE and RFA was 93.4%, the 1- and 3- year survival rates were 74.5% and 36.8% after TACE and RFA. The effective rate of PR + NC after TACE was 83.2%. The 1-, 3- and 5- year survival rates were 69.3%, 21.7%, 8.4% after TACE. The effective rate of PR + NC after TAI was 27.5%, the 1- and 2- year survival rates were 11.6% and 0% after TAI. The liver function, color-ultrasonography finding and alpha-fetoprotein after TACE + RFA, TACE and TAI were compared. There was no significant difference in each index between TACE and RFA or TACE as well as in liver function between TACE and RFA or between TACE and TAI.

CONCLUSION: The therapeutic effectiveness of TACE before or after hepatectomy is most significant, while the

INTRODUCTION

Transcatheter arterial interventional therapy is extensively used in treating hepatocellular carcinoma (HCC). To reduce the intra-hepatic recurrence and improve the protective efficacy after treatment for HCC, some scholars have proposed combined interventional therapy as a choice of treatment for HCC^[1]. We analyzed the therapeutic effectiveness of different combined interventional therapies for HCC according to the survival rate, liver function, image data and indications of tumor.

MATERIALS AND METHODS

Patients

A total of 1126 patients were retrospectively studied from January 1994 to October 2003. Of them, 873 were males and 253 were females (aged 32-76 years, mean 56.2 years). The patients were treated following the HCC Clinical Diagnosis and Staging Standard stipulated at the 8th National Academic Conference of Liver Cancer in September 2001. Among these patients, 78 had a family history of liver cancer, 985 had a medical history of serum hepatitis, 48 had a medical history of hepatitis C, 342 had cardinal symptoms of right upper quadrant pain, 336 felt epigastric distention after dinner or suffered from anorexia, 162 had low fever accompanied with progressing wasting, 286 exhibited no overt symptoms. Prior to the treatment, AFP was greater than 20 ng/mL in 735 cases, greater than 400 ng/mL in 521, negative in 227, unknown in 164. A total of 874 patients were followed up for 2

Table 1 Clinical data of 1126 patients

Group	Cases (n)	Size of tumor (cm)			Clinical staging			Liver function level		
		<5	5-10	>10	I	II	III	A	B	C
Preop. TACE	258	20	145	93	104	154	0	155	103	0
Postop. TACE	154	9	76	69	16	138	0	83	71	0
TACE + RFA	126	3	57	66	48	57	21	92	34	0
TACE	469	31	239	199	156	251	62	252	197	20
TAI	119	11	47	61	28	47	44	14	73	32
Total	1126	74	564	488	352	647	127	596	478	52

Table 2 Clinical data of 1126 patients

Group	Cases (n)	Karnofsky scores		HBsA	AVF		Embolus in PV	
		0-80	80-100	(+)	HV	PV	Right	Left
Preop. TACE	258	172	86	221	9	19	67	73
Postop. TACE	154	98	56	124	4	10	21	16
TACE + RFA	126	83	43	107	12	27	32	26
TACE	469	291	178	412	76	125	106	92
TAI	119	78	41	101	22	34	32	28
Total	1126	722	404	965	123	215	258	235

to 63 mo (averaged 26.3 mo). Combined interventional therapy was given, including transcatheter arteria hepatica chemoembolization (TACE) before or after hepatectomy, radio-frequency ablation (RFA) after TACE, Chinese medicine treatment and biotherapy after TACE or transcatheter arteria hepatica infusion (TAI). The clinical data of various kinds of combined interventional therapy for HCC are shown in Tables 1 and 2.

TACE

Twelve mg mitomycin (MMC) and 1.0 g 5-FU were diluted in 60 mL sodium chloride solution respectively and infused through a catheter. Then 50 mg epirubicin (EPI) and iodinated oil were compounded into a mixed emulsifier and infused into the blood-supply artery of the tumor through a catheter. Then, the artery was embolized with granules of spongia gelatinosa.

TAI

Fifty mg EPI, 12 mg MMC, and 1.0 g 5-FU were diluted in 60 mL sodium chloride solution respectively and infused through a catheter into the blood-supply artery of tumor. HCC patients treated 3-4 times with TACE at 1-2 mo intervals after hepatectomy, if pathological report showed remnant cancer cells on the cutting edge or AFP > 20 ng/mL or the image showed remnant tumor. Those who lost the chance of one-stage operation were treated with TACE and underwent re-examination of CT after 1-2 mo. If iodinated oil deposited in the tumor was reported to be coarctate, the volume of tumor was decreased or the normal liver had compensatory hyperplasia, the patients underwent hepatectomy after their recuperation. Those who did not recuperate or rejected hepatectomy were treated with RFA 3-4 wk after TACE. On the first day after TACE or TAI, the patients were treated with the following Chi-

Table 3 Accumulated survival rate for 874 HCC patients after various kinds of interventional therapy

Group	Cases (n)	1 yr		3 yr		5 yr	
		Cases (n)	Survival rate (%)	Cases (n)	Survival rate (%)	Cases (n)	Survival rate (%)
Preop. TACE	198	148/198	74.7	82/198	41.4	58/157	36.9
Postop. TACE	114	90/114	78.9	46/114	40.4	33/88	37.5
TACE + RFA	106	79/106	74.5	39/106	36.8	-	-
TACE	387	268/387	69.3	84/387	21.7	26/309	8.4
TAI	69	8/69	11.6	0/69	0	0/69	0
Total	874	593/874	67.8	251/874	28.7	117/623	18.8

nese herbal medicines: 30 g Milkvetch Root, 20 g Dangshen, 15 g Largehead Atractylodes Rhizome, 15 g Indian Bread, 30 g Huaishan, 15 g Finger Citron, 15 g Bupleurum chinense, 15 g Danshen Root, 3 g Radix Notoginseng, 15 g Virgate Wormwood Herb, 20 g Tianjihuang, 20 g Canton Love-Pea Vine, 5 g Radix Glycyrrhizae. The herbal medicines were decocted in water for oral dose once daily for 3-4 wk as a course of treatment. After 3-5 d, the patients received intramuscular injection of 1000 U interferon once a day for 20 d as a course of treatment.

Follow-up

The liver and renal function, AFP, blood RT, KPS scores were re-examined, and sonography, CT, chest X-Ray were performed once a month, and then every 3 mo during the follow-up period. The date on which the patients lost their follow-up was regarded as the date of death. According to the WHO evaluation standard of therapeutic effect of solid tumor (1981), the patients who were treated with TACE + RFA, TACE and TAI were categorized into 4 degrees: complete remission (CR), partial remission (PR), no change (NC) and progression (PD). The first 3 degrees were considered efficient and the last degree was considered inefficient.

Statistical analysis

SPSS12.0 was used for statistical analysis. The survival rate was evaluated by adopting probability ratio multiplication theorem, and chi-square test was used for comparison between the 2 groups. $P < 0.05$ was considered statistically significant.

RESULTS

A total of 874 cases were followed up for 1-7 years. Comparison of the accumulated survival rate after various kinds of combined interventional therapy is shown in Table 3. The therapeutic effect of interventional therapy in 562 patients is shown in Table 4. Changes of liver function, tumor blood-supply and AFP in 562 cases after interventional therapy were compared. Fifteen days after combined interventional therapy, color Doppler was used to detect the blood flow around and inside the tumor. The change in recovery rate of AFP before and after TACE + RFA, TACE and TAI was compared. The results are shown in Table 5.

Table 4 Therapeutic effect of TACE+RAF, TACE and TAI in 562 patients

Group	Cases (n)	PR	NC	PD	Effective rate (%)
TACE + RFA	106	73	26	7	93.4
TACE	387	276	46	65	83.2
TAI	69	8	11	50	27.5
Total	562	357	83	122	81.5

Table 5 Changes of indexes before and after interventional therapy n (%)

Group	Index			
	Degrade of Child liver function	Blackout of blood flow inside tumor	Blackout of blood flow around tumor	Recovery of AFP
TACE + RFA	33/106 (31.1)	59/106 (55.7)	54/106 (50.9)	49/106 (46.2)
TACE	119/387 (30.7)	212/387 (54.8)	189/387 (48.8)	174/387 (45.0)
TAI	23/69 (33.3)	0/69 (0)	0/69 (0)	0/69 (0)
Total	175/562 (31.1)	271/562 (48.2)	243/562 (43.2)	223/562 (39.7)

DISCUSSION

At present, hepatectomy remains the preferred method to treat primary liver cancer. Second-stage hepatectomy after TACE is a great breakthrough in the field of liver cancer treatment after hepatectomy of macro-hepatoma in the 1950-1960s and micro-hepatoma in the 1970s^[3]. TACE after radical excision of hepatoma can efficiently kill remnant cancer cells, decrease recurrence and increase survival rate remarkably^[4,5]. However; it was reported that TACE can damage hepatic and immunologic function, thus decreasing the survival rate^[6]. Zheng *et al*^[4] retrospectively analyzed the therapeutic results of 420 HCC patients after combined therapy, and found that the 1- and 3-year survival rates of non-angioencroached HCC patients after treated with TACE are 100% and 60% before radical excision, 77.8% and 22.2% before palliative excision, respectively, higher than those of the patients not treated with TACE before hepatectomy, and there is no significant difference between TACE before and after hepatectomy. Wang *et al*^[7] reported that the 1-, 3-, 5- year survival rates of 20 HCC patients after treated with combined therapy are 95.0%, 63.5%, 32.9% after second-stage hepatectomy, suggesting that the 1-, 3-, 5- year survival rates after TACE before or after hepatectomy are the highest. The reasons may be as follows: some of the 1126 cases treated with TACE before or after hepatectomy had hepatic AVF and embolus of hepatic portal vein, only interventional therapy was given before hepatectomy, and TACE treatment did not continue with double-chemoembolization or internal radiation. All these indicate that cancer cells can extend to distal ramulus along homonymous portal vein during operation procedures. Though macroscopic circumscription of hepatectomy is satisfactory, there are remnant cancer cells on the cutting edge, and liver cancer

complicating hepatic cirrhosis limits circumscription of hepatectomy. The above-mentioned factors are the major reasons for recurrence which is an important reason for the low survival rate after hepatectomy. For this reason, it is necessary to treat HCC patients with TACE combined with Chinese medicine and biotherapy to increase their immunologic function after hepatectomy. To effectively increase the survival rate, HCC patients should be treated with double-chemoembolization or internal radiation before hepatectomy.

Combined interventional therapy including TACE, TAI, RAF, percutaneous intra-tumor absolute alcohol injection (PEI), percutaneous intra-tumor acetic acid injection (PAAI), percutaneous intra-tumor hot saline water injection (PSI), percutaneous cryotherapy (PCT), is dominantly used in non-surgical treatment of HCC. Except for TACE and TAI, extra-hemal and circumscriptus "interstitial substance therapy"^[8] can treat HCC through different mechanisms. It was reported that chemoembolization can reduce the number of newly generated tumor vessels and the remnant tumor may generate new vessels through various ways^[9]. To increase the life span, we believe that HCC patients should be treated with RFA, PEI, PAAI, PSI, and PCT on the basis of TACE to kill remnant cancer cells. Liu *et al*^[10] reported that the 1-, 2-, 3- year survival rates of HCC patients after treated with TACE are 59.52%, 22.06% and 14.34% , while the 1- and 3- year survival rates of HCC patients after treated with TAI are 27.94% and 0%. In our study, the 1-, 2-, 3- year survival rates of 387 patients after treated with TACE were 69.3%, 21.7% and 8.4% respectively, and the 1- and 3- year survival rates of 69 after treated with TAI were 11.6% and 0%, suggesting that combined interventional therapy for HCC is influenced by many factors.

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RAPID COMMUNICATION

Primary pancreatic lymphoma: Report of six cases

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Abstract

AIM: To heighten recognition of primary pancreatic lymphoma (PPL) in clinical practice.

METHODS: A retrospective review of the clinical presentation, imaging characteristics and pathological features of PPL patients were presented, as well as their diagnosis and treatment, in combination with literature review.

RESULTS: Histological diagnosis was made in four patients by surgery and in two patients by EUS-FNA. The six PPL patients (5 males and 1 female; age range, 16-65 years; mean age, 46 years) had the duration of symptoms for two weeks to three months. The primary presenting symptoms, though not characteristic, were abdominal pain, abdominal masses, weight loss, jaundice, nausea and vomiting. One of the patients developed acute pancreatitis. In one patient, the level of serum CA19-9 was 76.3 µg/L. Abdominal CT scan showed that three of the six tumors were located in the head of pancreas, two in the body and tail, and one throughout the pancreas. Diameter of the tumors in the pancreas in four cases was more than 6 cm, with homogeneous density and unclear borders. Enhanced CT scan showed that only the tumor edges were slightly enhanced. The pancreatic duct was irregularly narrowed in two cases whose tumors were located in the pancreatic head and body, in which endoscopic retrograde cholangiopancreatography (ERCP) showed that the proximal segment was slightly dilated. Two patients underwent Whipple operation, one patient underwent pancreatectomy, and another patient underwent operative biliary decompression. PPL was in stage I E in 2 patients and in stage II E in 4 patients according to the Ann Arbor classification system. The diagnosis of B-cell non-Hodgkin's lymphoma was made in all patients histopathologically. All six patients underwent systemic chemotherapy, one of whom was also treated

with gamma radiometry. One patient died two weeks after diagnosis, two patients lost follow-up, two patients who received chemotherapy survived 49 and 37 mo, and the remaining patient is still alive 21 mo, after diagnosis and treatment.

CONCLUSION: PPL is a rare form of extranodal lymphoma originating from the pancreatic parenchyma. Clinical and imaging findings are otherwise not specific in the differentiation of pancreatic lymphoma and pancreatic cancer, which deserves attention. EUS-guided fine-needle aspiration (EUS-FNA) of the pancreas requires experienced cytopathologists as well as advanced immunohistochemical assays to obtain a final diagnosis on a small amount of tissue. Surgery and adjuvant chemotherapy or radiotherapy can produce fairly good outcomes.

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Key words: Pancreatic malignant tumor; Lymphoma; non-Hodgkin's lymphoma; Diagnosis

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INTRODUCTION

Lymphoma includes Hodgkin's and non-Hodgkin's forms. Hodgkin's lymphomas rarely disseminate to extra-lymphatic organs, while non-Hodgkin's lymphomas often invade extra-lymphatic organs. Most primary pancreatic lymphomas (PPL) are non-Hodgkin's Lymphomas. More than 25 percent of non-Hodgkin's lymphomas originate from extra-lymphatic organs, about 30 percent of which may involve the pancreas^[1]. Isolated PPL is quite rare, less than 1 percent^[2]. In a review of 207 cases of malignant pancreatic tumors, there were only three cases (1.5%) of pancreatic lymphoma^[3]. In fine-needle aspiration (FNA) of 1050 cases of pancreatic lesions, Volmar *et al*^[4] only found 14 cases (1.3%) of PPL. Clinically, PPL is most likely to be misdiagnosed as pancreatic cancer. The present article is a retrospective review of six cases of PPL, based on which the clinical presentation, imageologic characteristics and pathologic features of PPL are discussed in the context of the world literature in an attempt to heighten clinicians' awareness of the condition.

MATERIALS AND METHODS

Patients

Six cases of PPL were identified and treated in Department of Gastroenterology, Changhai Hospital, Second Military Medical University, Shanghai, China, during a 15-year period from January 1990 to June 2005. The clinical characteristics of all the patients were consistent with the criteria for the diagnosis of PPL defined by Behrns *et al*¹¹. On physical examination, no enlarged superficial lymph nodes were found; no enlarged diaphragmatic lymph nodes on chest X-ray films were found either. There was no change of WBC classification on hemograms; there were no significant hepatic and splenic foci. However, there was evidence of a pancreatic mass on laparotomy, and the lymph nodes involved were confined to areas around the pancreas.

Methods

A retrospective review of the clinical data revealed that malignant lymphoma was unanimously the initial diagnosis of all the six cases. Immunohistochemical analysis by EnVision-methods included leukocyte common antigen (LCA), CD20, CD34, CD68, Chr, CD45RO and Ki-67, of which LCA indicates that tumor cells come from lymphocytes, CD20 is the marker of B-cell, and CD45RO is the marker of T-cell.

All patients were staged according to the Ann Arbor staging system for non-Hodgkin's lymphomas, stage I: invasion of lymphoma is confined to the pancreas; stage II: apart from pancreatic invasion, there is also invasion of local lymph nodes; stage III: apart from pancreatic invasion, the focus also infiltrates the upper diaphragm; and stage IV: apart from pancreatic invasion, there is generalized infiltration¹⁵.

RESULTS

Clinical presentation

The six PPL patients included five males and one female who ranged in age from 16 to 65 years with a mean of 46 years, and whose duration of symptoms ranged from two weeks to three months. The primary presenting symptoms were abdominal pain, an abdominal mass, weight loss, jaundice, nausea and vomiting, which were similar to what were reported in the overseas literatures¹⁶⁻⁹¹, except that there were no lower back pain, fever and chill, ascites and hemorrhage of the upper digestive tract (Table 1).

Laboratory and imaging findings

WBC count was within the normal range in all six cases. In four patients aspartate aminotransferase (ALT) was 80-265 U/L and alkaline phosphatase (AKP) was 120-216 U/L; in three patients, the level of serum bilirubin was 42-216 $\mu\text{mol/L}$ and CA19-9 was 76.3 $\mu\text{g/L}$. Liver function was otherwise normal. Abdominal CT scan demonstrated that the tumor was located in the head of pancreas in three cases, in the body and tail in two cases, and in the whole pancreas in the remaining case. The tumors were larger than 6 cm in four cases, with almost homogeneous density and unclear edges. Enhanced CT scan only enhanced

Table 1 Clinical presentation of PPL *n* (%)

Data	This report	Overseas report ^[5]	Overseas CDI ^[91]
Case number	6	11	85
Abdominal pain	5 (83)	9 (82)	62 (73)
Weight loss	3 (50)	4 (36)	43 (51)
Abdominal mass	2 (33)	-	-
Jaundice	2 (33)	2 (18)	36 (42)
Nausea	2 (33)	2 (18)	29 (34)
Vomiting	1 (17)	-	15 (18)
Lower backache	-	3 (27)	-
Fever	-	2 (18)	6 (7)
Ascites	-	1 (9)	-
Hemorrhage of UDT	-	-	2 (2)

CDI: Compositive details index; UDT: Upper digestive tract.

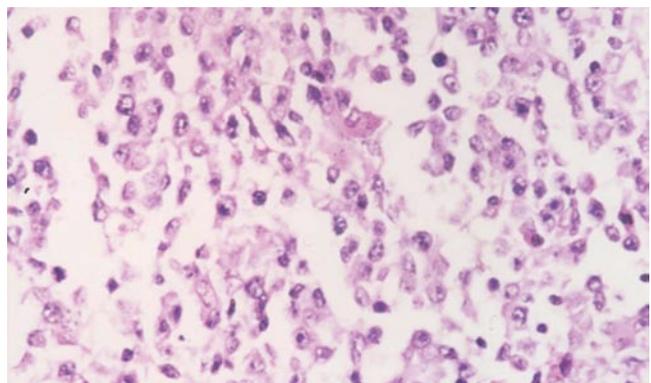


Figure 1 Cytologically, lymphoma cells are round, oval, with prominent and large nucleus, arranged diffusely with obvious heterogeneity (Hematoxylin-eosin stain; original magnification $\times 200$).

the edges slightly. ERCP in two cases revealed that the pancreatic duct at the body of the head was irregularly narrowed, the proximal segment of which was slightly dilated.

Histopathologic study

The specimens were obtained from pancreatic resection in four cases, and from EUS-guided fine-needle aspiration (EUS-FNA) in the remaining two cases. Microscopic examination showed that the lymphoma cells were round or elliptic, and arranged diffusely, with little plasma, thick karyotheca, thick chromatin and clear nuclei. Immunohistochemical analysis showed LCA (+), CD20 (+), CD34 (-) and CD68 (-). Histopathological examination confirmed the diagnosis of the six cases as B cell pancreatic lymphoma (Figures 1-3).

Diagnosis, treatment and prognosis

Of the six patients, diagnosis was confirmed by pathological study of the pancreatic tissue resected by EUS-FNA in two patients, by my cytological study in one patient, and by general skeleton ECT scan in another patient. One patient underwent biliary-intestinal anastomosis. According to Ann Arbor classification, two cases belonged to I E stage, and the remaining four cases to II E stage. All six patients underwent chemotherapy, one of whom also underwent linear accelerator radio-

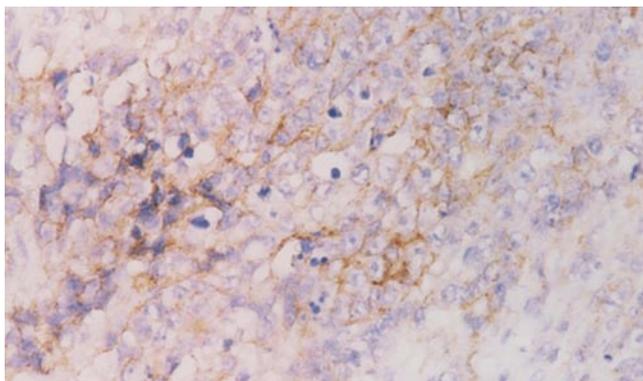


Figure 2 Immunohistochemical analysis showing CD20 (+) (EnVision's stain; original magnification $\times 200$).

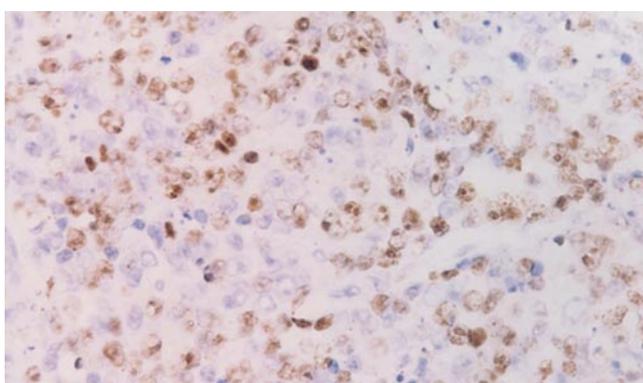


Figure 3 Immunohistochemical analysis showing Ki-67 (+) (EnVision's stain; original magnification $\times 200$).

therapy, and another underwent stereotaxic γ -knife radiotherapy. One patient died two weeks after the diagnosis was confirmed; two patients were lost to follow-up; two patients survived for 49 and 37 mo, and the remaining one patient is still being followed up.

DISCUSSION

Primary pancreatic lymphoma (PPL) is an extremely rare disease which occurs in pancreatic situ, with or without involvement of peripancreatic lymph nodes. The clinical manifestation and imaging result of PPL resemble other pancreatic occupying lesions like pancreatic carcinoma. However, unlike carcinomas, PPLs are potentially treatable even if not found at early stage. PPL only accounts for fewer than 2% of extra-nodal malignant lymphomas and 0.5% of cases of pancreatic masses^[10,11]. To date, only fewer than 150 and 20 cases of PPL have been reported in English literature^[12] and Chinese medical literature, respectively. In China, from 1995 to 2003 there were totally 16 cases of PPL reported^[13]. The data show a strong male predominance (male to female ratio of 13:3) and increased trend with age (median age of 57.5 years), which is similar to the present study. However whether the mean age of PPL is older than that of pancreatic carcinoma remains controversial.

Although the clinical presentation of PPL is varied, some findings may support PPL rather than pancreatic

cancer. Bellyache and abdominal mass are two major symptoms which present in 83% and 58% of PPL cases, respectively^[12]. Two of six of our patients (33%) presented abdominal mass while Yu *et al* reported that only 1.3% of patients with adenocarcinoma presented abdominal mass^[14]. The other symptoms are jaundice, reflux, weight loss, bowel obstruction and diarrhea^[6,15]. Interestingly there are almost 12% of PPL mimicking acute pancreatitis. It is noticeable that one in six of our patients presented acute pancreatitis. Obstructive jaundice was found to be less frequent than in pancreatic cancer^[16]. While the common symptoms in non-Hodgkin's lymphoma like fever, chills and night sweats were rare in PPL, making it hard to diagnose only depending on the signs^[17]. The majority PPLs occur in the head of pancreas, though tumor could also be found in the body and tail regions^[7]. It was reported that over half of PPL patients presented with an epigastric mass and the diameter was bigger than 6 cm in 70% of patients of PPL^[18]. The laboratory test is non-specific for diagnosis of PPL. Our data reveal that serum carbohydrate antigen 19-9 (CA19-9) level in PPL patients was normal or slightly elevated. This is different from pancreatic adenocarcinoma, in which almost eighty percent of patients get a higher CA19-9 level. The abnormality of serum ALT, AKP, and bilirubin was secondary to the bile duct obstruction.

Imaging results play a key role in diagnosis of PPL. Percutaneous ultrasound (US), endoscopic ultrasonography (EUS), computed tomography (CT) and MRI are well-established procedures to evaluate pancreatic masses^[19,20]. Previously 16 Chinese PPL cases could all be found pancreatic masses by CT or US before therapy, with or without signs of peripancreatic lymph node invasion. CT is by far the most common imaging technique used in the detection and characterization of pancreatic tumors. The picture of PPL on CT resembled that of pancreatic carcinoma, including enlargement of pancreatic head and density changes. However, there are fewer signs of invaded large vessel and metastasis of the liver and spleen. There are fewer chances to find pancreatic duct dilation in PPL when compared with pancreatic cancer. Merkle *et al*^[9] reported the combination of a bulky localized tumor in the pancreas without significant dilation of the main pancreatic duct strengthens a diagnosis of pancreatic lymphoma over adenocarcinoma. Furthermore, if enlarged lymph nodes are encountered below the level of the renal veins, virtual exclusion of adenocarcinoma is possible. While Arcari *et al*^[17] insisted that imaging techniques could suggest the suspicion of PPL but are unable to distinguish PPL from pancreatic adenocarcinoma. Therefore, the final diagnosis of PPL should depend on histopathologic examination. In four of six PPL patients in current study the tissue was obtained during operation and in the other two cases the tissue was obtained by EUS-FNA. Most reported Chinese PPL cases were finally diagnosed by surgeons while there will be more studies using FNA in the future. However, when compared with surgery, it is difficult to obtain enough specimen by FNA to perform immuno-histochemical analysis and may lead to false-negative result^[7]. Di Stasi *et al*^[21] reported that CT or US guided FNA is a safe and rapid technique to make

histological diagnosis by which we could get more tissues than by EUS. However during CT scan it is difficult for operators to supervise the needle path. EUS-guided tissue sampling of pancreatic masses is the superior method because it is dynamic and real-time. EUS can clearly reveal the boundary of tumor and color doppler flow imaging(CDFI) can show the vein path, sequentially guide the needle aspiration and avoid the vessel damage^[22,23]. O'Toole *et al*^[24] reported the complication rate of EUS-FNA was as low as 1.6%, indicating it is a safe method. Most importantly, FNA depending methods should be further applied because it is helpful to avoid unnecessary surgery.

Totally four in six of our patients underwent operation. Among them two cases were treated with Whipple surgery, one by distal pancreatectomy and another by operative biliary decompression. The reason for operation is that the definitive diagnosis was not made before surgery. If these patients had been detected using FNA, the exploration could have been skipped. Thus the treatment strategy could have been direct chemotherapy or radiotherapy. Unfortunately, all published 16 Chinese PPL cases were treated with surgery, which made the final diagnosis. It can be predicted that the more frequent usage of FNA, including either CT or EUS guided-FNA may minimize the number of operations for PPL. Bouvet *et al*^[8] reported ten in eleven PPL patients, have been performed explorative surgery, only in three of them the tumor could be fully resected. Among eight unresected cases, seven cases were treated with combined CHOP chemotherapy and radiotherapy with dosage of 30 to 45 Gy. The median survival time was 67 mo (11-191 mo). Behrns *et al*^[1] reported that the median survival time for single chemotherapy and radiotherapy-treated PPL patients was 13 and 22 mo, respectively. While the survival time could be improved to 26 mo if combining medication with radiotherapy. Therefore, the first choice for PPL treatment should be combination of chemotherapy and radiotherapy, rather than surgery. With the advancement of techniques, surgery seems to be only effective when FNA is not available or diagnosis can not be made on histology. It has already been proved that single pancreas resection could not improve the survival rate of PPL but cause more complications. However, the PPL patient with biliary tract or gastrointestinal obstruction should be performed biliary or gastric bypass to relieve the symptoms.

In conclusion, PPL is a rare disease with non-specific symptoms, laboratory tests and imaging examination results. Cytology or tissue histology is fundamental for diagnosis and chemo- or radiotherapy is preferred for treatment. FNA technique is recommended as a routine examination, while total pancreatectomy is considered to have no impact on survival and with its associated morbidities, is not generally recommended for diagnosis and treatment of PPL. As a result, PPL will not be such a disease with poor prognosis in the future.

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RAPID COMMUNICATION

Indwelling catheter and conservative measures in the treatment of abdominal compartment syndrome in fulminant acute pancreatitis

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Abstract

AIM: To study the effect of combined indwelling catheter, hemofiltration, respiration support and traditional Chinese medicine (e.g. Dahuang) in treating abdominal compartment syndrome of fulminant acute pancreatitis.

METHODS: Patients with fulminant acute pancreatitis were divided randomly into 2 groups of combined indwelling catheter celiac drainage and intra-abdominal pressure monitoring and routine conservative measures group (group 1) and control group (group 2). Routine non-operative conservative treatments including hemofiltration, respiration support, gastrointestinal TCM ablation were also applied in control group patients. Effectiveness of the two groups was observed, and APACHE II scores were applied for analysis.

RESULTS: On the second and fifth days after treatment, APACHE II scores of group 1 and 2 patients were significantly different. Comparison of effectiveness (abdominalgia and burbulence relief time, hospitalization time) between groups 1 and 2 showed significant difference, as well as incidence rates of cysts formation. Mortality rates of groups 1 and 2 were 10.0% and 20.7%, respectively. For patients in group 1, celiac drainage quantity and intra-abdominal pressure, and hospitalization time were positively correlated ($r = 0.552, 0.748, 0.923, P < 0.01$) with APACHE II scores.

CONCLUSION: Combined indwelling catheter celiac drainage and intra-abdominal pressure monitoring, short veno-venous hemofiltration (SVVH), gastrointestinal TCM ablation, respiration support have preventive and treatment effects on abdominal compartment syndrome of fulminant acute pancreatitis.

INTRODUCTION

There are certain guidelines for treatment of severe acute pancreatitis (SAP). However, about 11% of SAP patients suffer from the complication of abdominal compartment syndrome (ACS), and about 25% of SAP patients are fulminant acute pancreatitis (FAP). Incidence rate of ACS is higher in FAP, and its mortality rate is as high as 60%. Up till now, there have been no standard treatments for ACS^[1,2]. In the present study, we used combination of celiac indwelling catheter drainage and intra-abdominal pressure monitoring, several short veno-venous hemofiltration (SVVH), respiration support and gastrointestinal TCM ablation for treatment and predicting patient's conditions of ACS in FAP. Through comparison with the control group, we demonstrate that the combined therapy is effective for treatment of ACS.

MATERIALS AND METHODS

Patients

A total of 110 FAP patients were received and treated in First Aid Center and Hepato-biliary Surgery Department of Affiliated Hospital of Guiyang Medical College^[3,4]. When they were hospitalized, the cumulative scorings of CT serious index (CTSI), APACHE II and SAP were 7.85 ± 1.10 , 17.51 ± 4.51 and grade II respectively.

Methods

Patients were divided randomly into groups of indwell-



Figure 1 Indwelling catheter. (✓) Celiac drainage; (✗) Intra-abdominal pressure detection.

ing catheter celiac drainage and intra-abdominal pressure monitoring and routine non-operative conservative treatment measures (Figure 1) group (group 1, 45 cases) and control group (group 2, 65 cases). There was no significant difference ($P > 0.05$) in gender, age, cumulative scorings of CTSI and APACHE II between the two groups. Routine non-operative conservative treatment measures, including SVVH, gastrointestinal TCM ablation, respiration support and drug therapy, were conducted in group 2 patients. For group 1 patients centesis in right side or two sides of abdominal cavity, installation of indwelling catheter for continuous drainage (drain quantity was recorded daily) were conducted on the first day of hospitalization, and intra-abdominal pressure was monitored and recorded on the first, second and fifth days since installation of indwelling catheter. The decision of time for hemofiltration was based on the indications of systemic inflammatory response syndrome (SIRS). On the day of hospitalization Dahuang or Qingyitang was infused by gastric canal or anus drip (3 times daily).

Observation of clinical effectiveness: Abdominalgia, burbulence time, hospitalization time for groups 1 and 2 were observed. Celiac drainage and intra-abdominal pressure were monitored for group 1; APACHE II cumulative scores before treatment, on the second and fifth days after treatment in groups 1 and 2 were recorded.

Statistical analysis

Data are expressed as mean \pm SD. SPSS 12.0 was used for statistical analysis. $P < 0.05$ means significant.

RESULTS

Patients' conditions

During hospitalization and treatment period, there was no significant difference in the two groups for cumulative scorings of CTSI and APACHE II. On the second and fifth days after treatment with combined indwelling catheter drainage, gastrointestinal TCM ablation, SVVH, respiration support and drugs, the cumulative scorings of APACHE II in group 1 were significantly lower than in group 2 ($P < 0.01$); cumulative scorings of APACHE II were significantly decreased compared with before treatment ($P < 0.01$, Table 1).

Table 1 Patient's conditions of severity transformation (mean \pm SD)

Group	State of illness when hospitalized		Change of APACHE II during treatment	
	CTSI scoring	APACHE II scoring	2 d after treatment	5 d after treatment
1	7.61 \pm 0.67	16.44 \pm 2.28	9.66 \pm 1.88 ^b	4.63 \pm 1.46 ^b
2	7.59 \pm 0.86	15.74 \pm 1.91	13.46 \pm 1.93	10.78 \pm 2.01

^b $P = 0.000$ vs group 2.

Table 2 Celiac drainage and intra-abdominal pressure (mean \pm SD)

	1st	2nd	3rd	<i>P</i>
Drain quantity (mL)	1817 \pm 639	815 \pm 423 ^a	85 \pm 40 ^a	0.000
IAP (cmH ₂ O)	29.29 \pm 5.53	13.95 \pm 4.05 ^b	6.71 \pm 1.68 ^b	0.000
APACHE II scorings	16.44 \pm 2.28	9.66 \pm 1.88	4.63 \pm 1.46	0.000

^a $r = 0.55$ vs IAP, ^b $r = 0.92$ vs APACHE II scorings.

Table 3 Local symptoms and treatment effect (mean \pm SD)

Group	Relief time		Treatment effect		
	Abdominalgia (d)	Burbulence (d)	Hospitalization (d)	Mortality rate (%)	Rate of cyst (%)
1	3.27 \pm 0.87 ^b	6.90 \pm 1.18 ^b	15.59 \pm 3.89 ^b	10.0 ^b	8.9 ^b
2	14.13 \pm 2.14	23.36 \pm 3.76	28.28 \pm 4.61	20.7	37.9

^b $P < 0.01$ vs group 2.

Celiac drainage and intra-abdominal pressure

Drains of 45 cases in group 1 were all dematiaceous bloody liquid. Drainage period was 3.5 ± 0.85 d; drain quantity was positively correlated ($r = 0.552$, $P < 0.01$) with intra-abdominal pressure (IAP) ($r = 0.552$, $P < 0.01$). While IAP was positively correlated with cumulative scorings of APACHE II ($r = 0.748$, $P < 0.01$, Table 2).

Relief time for abdominalgia and burbulence, and hospitalization time

The relief time of abdominalgia, burbulence, and hospitalization time in group 1 were significantly shorter than those in group 2 ($P < 0.01$). Mortality rates in group 1 were decreased compared to group 2, with no significant difference. Incidence rates of cysts in group 1 were significantly decreased compared to group 2 ($P < 0.01$, Table 3).

DISCUSSION

FAP is characterized by rapid deterioration of patient's conditions. Multi-organ (specially pancreas and gastrointestinal tract) dysfunction appeared in early stage.

ACS in FAP is divided into four grades according to IAP: first grade is 10-14 cm H₂O, second grade is 15-24 cm H₂O, third grade is 25-35 cm H₂O, and fourth grade is > 35 cm H₂O. In group 1 of our experiment, celiac intra-abdominal pressure of 45 cases was 29.29 ± 5.53 cm H₂O, diagnosed as ACS clinically^[4-6]. Currently, ACS is detected mainly by bladder manometry method, which is an indirect

method. However, there are certain influencing factors. Therefore we used indwelling catheter celiac laying canal to directly detect intra-abdominal pressure, and performed canal drainage. It could prevent celiac dropsy ACS, avoid disturbance of celiac function when operating, and the effect of anesthesia on laparotomy and celiac operation, and consequently prevent ACS^[7-12]. In our study, the combined approach of indwelling catheter drainage, SVVH and gastrointestinal TCM ablation, respiration support and use of other drugs was employed to treat ACS in FAP. We found that drain quantity was positively correlated with intra-abdominal pressure ($r = 0.55$), and intra-abdominal pressure was correlated with hospitalization time and APACHE II cumulative scorings ($r = 0.75, 0.92$). In comparison of effectiveness, that of the group 1 was significantly better than that of group 2 regarding abdominalgia disappearing time, burbulence relief time, and hospitalization time ($P = 0.000$); mortality rates were 10% and 20.7% in group 1 and 2, respectively; incidence rates of cysts between the two groups were significantly different ($P = 0.001$). The reasons for the better effect of group 1 might be that: (1) indwelling catheter drainage eliminated ACS caused by celiac dropsy; (2) hemofiltration or drainage improved paralysis of gastrointestinal tract caused by a variety of inflammatory cytokines, inflammation mediators, all kinds of enzymes and necrosis materials (including large, moderate and small molecular weight materials). As a consequence, damage of tissues and organs in dropsy (mesentery, epiploon, gastrointestinal and parietal peritoneal membrane) type of ACS was greatly reduced; (3) It was eliminated that the celiac disturbance and the effect on systemic multi-organs (especially gastrointestinal tract) caused by laparoscopic operation or laparotomy and anesthesia; (4) TCM, eg Da-huang could effectively reduce intestinal tract endotoxin and bacterial shift, alleviate intestinal mucosal membrane damage, and facilitate gastrointestinal movement and emptying^[9,13-16]. As far as we know, such study has not been reported.

In summary, combined indwelling catheter celiac drainage, intra-abdominal pressure monitoring, multi-SVVH, gastrointestinal TCM ablation, respiration support and use of drugs can prevent and treat ACS in FAP effectively. However, the problem of slow speed of indwelling catheter celiac drainage has still to be resolved^[13].

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Transhepatic catheter-directed thrombolysis for portal vein thrombosis after partial splenic embolization in combination with balloon-occluded retrograde transvenous obliteration of splenorenal shunt

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Abstract

A 66-year-old woman underwent partial splenic embolization (PSE) for hypersplenism with idiopathic portal hypertension (IPH). One week later, contrast-enhanced CT revealed extensive portal vein thrombosis (PVT) and dilated portosystemic shunts. The PVT was not dissolved by the intravenous administration of urokinase. The right portal vein was cannulated *via* the percutaneous transhepatic route under ultrasonic guidance and a 4 Fr. straight catheter was advanced into the portal vein through the thrombus. Transhepatic catheter-directed thrombolysis was performed to dissolve the PVT and a splenorenal shunt was concurrently occluded to increase portal blood flow, using balloon-occluded retrograde transvenous obliteration (BRTO) technique. Subsequent contrast-enhanced CT showed good patency of the portal vein and thrombosed splenorenal shunt.

Transhepatic catheter-directed thrombolysis combined with BRTO is feasible and effective for PVT with portosystemic shunts.

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Key words: Portal vein thrombosis; Idiopathic portal hypertension; Partial splenic embolization; Portosystemic shunts; Transhepatic catheter-directed thrombolysis; Balloon-occluded retrograde transvenous obliteration

Nakai M, Sato M, Sahara S, Kawai N, Kimura M, Maeda Y, Ibata Y, Higashi K. Transhepatic catheter-directed thrombolysis for portal vein thrombosis after partial splenic em-

INTRODUCTION

Acute portal vein thrombosis (PVT) after partial splenic embolization (PSE) in portal hypertension is a rare event^[1-3]. Stagnant splenic blood flow after PSE appears to result in PVT^[4,5]. Surgical treatment is generally ineffective and yields poor results. Consequently, direct percutaneous intervention is increasing in popularity as a therapeutic alternative. Portal systemic shunts (PSS) are frequently formed due to portal hypertension in patients with chronic liver diseases such as liver cirrhosis or idiopathic portal hypertension (IPH). Balloon-occluded retrograde transvenous obliteration (BRTO) is an effective treatment for gastric varices and encephalopathy with PSS^[6-8].

We report a patient with portal vein thrombosis after PSE in IPH with extensive portal systemic shunts treated with transhepatic catheter-directed thrombolysis combined with BRTO restoring portal blood flow.

CASE REPORT

A 66-year-old woman with a medical history of IPH was hospitalized with continuously bloody stool (melena). The HBV and HCV markers were all negative. IPH was diagnosed by liver biopsy and CT scan. Upper endoscopy was performed after admission and small esophageal varices without bleeding were pointed out. Telangiectasia was found in the transverse colon by colonoscopy and hemostasis was accomplished by endoscopic high-frequency coagulation. Despite repeated transfusion of red blood cells and platelets, laboratory examination revealed significant pancytopenia, with a white blood cell count of $1.1 \times 10^9/L$, red blood cell count of $2.71 \times 10^{12}/L$, and platelet count of $2.5 \times 10^{10}/L$. US and CT revealed splenomegaly.

The patient underwent PSE to reduce hypersplenism and portal hypertension. The splenic artery was catheter-

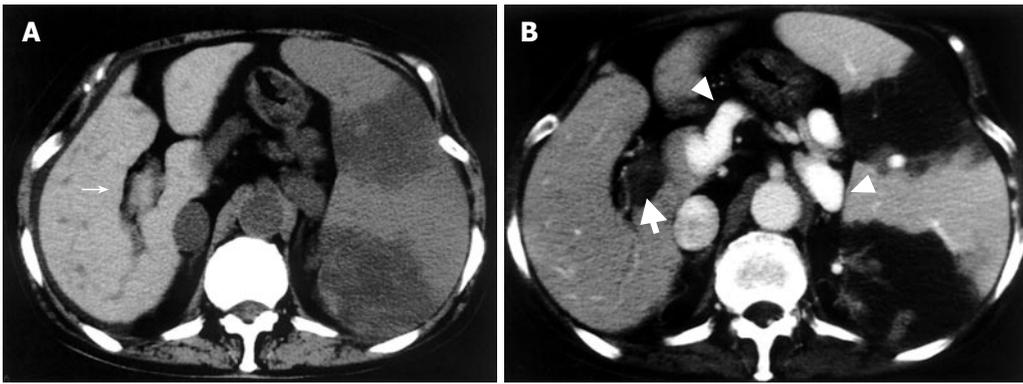


Figure 1 Abdominal CT one week after PSE. **A:** Plain CT showing high density lesions in the main portal vein and the 1st right branch (narrow arrow); **B:** Contrast-enhanced CT revealing no enhancement of portal vein indicating portal thrombosis (thick arrow) and portosystemic shunts (arrow head). Splenic infarction after PSE was also visualized.



Figure 2 No visualization of main portal vein due to extensive thrombosis (narrow arrow) and visualization of portosystemic shunts such as dilated left gastric vein and splenorenal shunt draining into left renal vein (thick arrow) on percutaneous transhepatic portography.

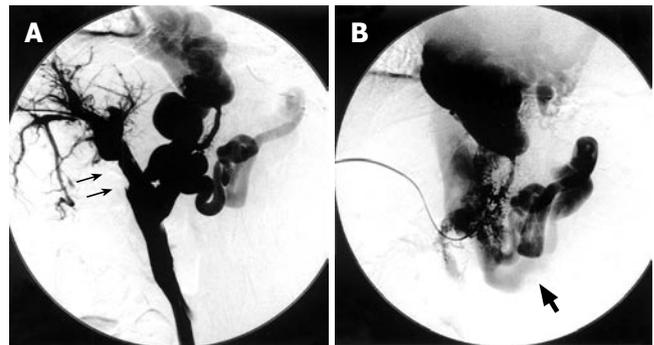


Figure 3 Percutaneous transhepatic portography after catheter-directed thrombolysis. **A:** Dissolution of the thrombus in portal vein (narrow arrow); **B:** Hepatofugal flow into dilated portosystemic shunts (thick arrow: left renal vein).

ized selectively *via* the femoral route, and the tip of microcatheter was inserted in a peripheral branch of the splenic artery. Small particles (1 mm × 1 mm × 1 mm) of gelatin sponge soaked in antibiotics were injected to achieve PSE. The platelet count rose to $14 \times 10^{10}/L$ on the 7th d after PSE. Plain CT revealed a low-density area in the spleen and high-density thrombus in the main portal vein and the 1st right and left branches (Figure 1A), and contrast-enhanced CT revealed no enhancement of the portal vein indicating extensive portal thrombosis (Figure 1B). Portosystemic shunts including a dilated left gastric vein to the paraesophageal vein and splenorenal shunt draining into the left renal vein were also visualized. Thrombolytics and anticoagulants were initiated, urokinase (240 000 U/d) and heparin (10 000 U/d) were given intravenously for 3 d. However, CT after systemic thrombolysis revealed that the thrombosis did not resolve.

It was therefore decided to attempt catheter-directed thrombolysis. With the patient under local anesthesia, a branch of the right portal vein was punctured with an 18-gauge needle under ultrasonic guidance. A 0.035-inch guidewire was advanced into the portal vein through the thrombus. A 4 Fr. straight catheter (Cook, Bloomington, IN) was advanced into the splenic vein and portography was performed. Percutaneous transhepatic portography (PTP) yielded no visualization of the main portal vein but did show large collaterals of the dilated gastric vein and splenorenal shunt (Figure 2). Urokinase at a dosage of 320 000 U was initially infused directly into the thrombus for 30 min. However, because immediate resolution of the thrombus was not observed, an hourly dose of 20 000 U of urokinase and a daily dose of 10 000 U of heparin



Figure 4 PTP after BRTO. BRTO was attempted to occlude splenorenal shunt in order to increase portal blood flow. A 7Fr balloon catheter (arrow) was advanced into the outflow pathway of the splenorenal shunt. A total of 10 mL of 5% ethanolamine oleate iopamidol (EOI) was injected and remained stagnant for a day during balloon occlusion.

were continuously infused into the thrombus. Two days later, PTP revealed total resolution of the thrombus in the portal vein and recirculation of portal blood flow to liver parenchyma (Figure 3A). However, there was still marked hepatofugal flow into the dilated PSS, inducing stagnation of portal blood flow (Figure 3B). BRTO was then attempted to occlude the splenorenal shunt in order to increase portal blood flow. An 8 Fr. J-type sheath (J Sheath; Medikid, Tokyo, Japan) was inserted into the left renal vein *via* the right femoral vein. A 7 Fr. balloon catheter (Selecon Cobra-shape MP catheter with 13 mm diameter balloon; Clinical Supply, Gifu, Japan) was advanced through the sheath into the outflow pathway of the splenorenal shunt (Figure 4). Balloon-occluded retrograde venography was carried out to identify the splenorenal shunt and the other outflowing vessels. The amount of sclerosing solution was

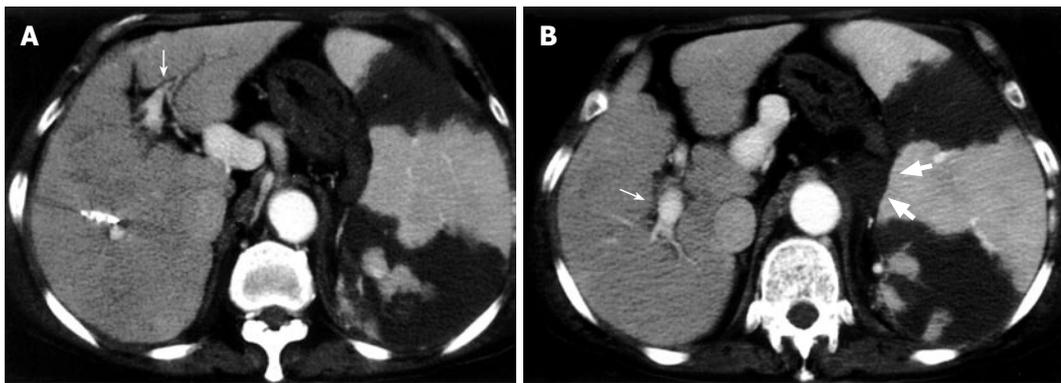


Figure 5 Contrast-enhanced CT 1 wk after catheter-directed thrombolysis and BRTO. **A:** Good patency of the portal vein (narrow arrow); **B:** Thrombosed splenorenal shunt (thick arrow).

determined from the volume of contrast material needed to fill these vessels. Preoperatively, 4000 U of haptoglobin (Mitsubishi Pharma, Osaka, Japan) was administered intravenously by drip infusion for the purpose of preventing a renal damage by hemolysis. A total of 10 mL of 5% (50 g/L) ethanolamine oleate iopamidol (EOI) was injected and remained stagnant in these vessels for a day during balloon occlusion. The following day, the balloon catheter was withdrawn after thrombosis of the splenorenal shunt was confirmed on contrast-enhanced CT (Figures 5A and B). The transhepatic catheter was subsequently removed using hemostatic microcoils for the transparenchymal tract without hemorrhagic complications. Six-month follow-up CT revealed good patency in the main portal vein.

DISCUSSION

Maddison^[9] first reported splenic embolization for hypersplenism in 1973. Spigos *et al*^[10] advocated partial splenic embolization (PSE) as an alternative method for the treatment of portal hypertension in 1979. PSE has been widely used for the treatment of portal hypertension and hypersplenism caused by IPH and liver cirrhosis since then^[1-3,9-11]. The aim of PSE is to decrease splenic blood flow and prevent platelet destruction in the spleen. An optimal increase in platelet count could be observed if a single procedure induces an infarct size of at least 60%-80% of the total spleen volume^[2,11]. Complications of PSE listed in the literature include local pain, ascites, pleural effusion, splenic abscess and also portal vein thrombosis (PVT) secondary to reduced blood flow in the splenic vein^[1-3]. Romano *et al*^[2] reported that no cases of PVT have been observed after PSE in patients with IPH. N'Kontchou *et al*^[3] reported that the incidence of PVT after PSE is 6.3% in patients with cirrhosis. Eguchi *et al*^[4] reported that the incidence of PVT after splenectomy is 25.0% in patients with IPH. PVT can occur with a higher incidence after splenectomy than after PSE.

PVT can occur due to other conditions including liver cirrhosis, IPH, direct invasion of neoplasm, hypercoagulable states, and inflammatory diseases such as pancreatitis and ulcerative colitis^[5,12,13]. PVT can result in intestinal ischemia and venous infarction in the acute stage. In the chronic stage, signs of portal hypertension such as esophageal varices, ascites, intestinal bleeding, liver atrophy, and liver failure become prominent^[12,13]. Management of acute

portal vein thrombosis is thus important. In our case, reduction of splenic blood flow due to PSE and outflow of the superior mesenteric vein (SMV) into the dilated PSS caused portal vein thrombosis.

The standard treatment for PVT is systemic thrombolysis with anticoagulants and local thrombolysis using interventional radiology (IVR) procedures^[13]. Urokinase (UK) has been used as a thrombolytic agent for PVT, but an enormous amount of UK is required for systemic use^[14], and often has no effect, as in our case. There are two IVR procedures to treat PVT: trans-arterial thrombolysis *via* the superior mesenteric artery (SMA) and catheter-directed thrombolysis^[15]. The angiographic method of trans-arterial thrombolysis is easy and involves only placement of a catheter into the SMA *via* the femoral artery^[16-18]. However, in our patient with remarkable portosystemic collaterals, UK would drain into hepatofugal collaterals and would not reach PVT easily. Local thrombolysis should be more effective for patients with PSS, since high concentrations of UK are directly infused into the PVT through a catheter. Access to catheter-directed thrombolysis is divided into two routes: the transhepatic route^[19-21] and the transjugular route^[22-24]. Uflacker^[24] emphasizes the advantages such as reduction of the possibility of intraabdominal bleeding and creation of adequate portal vein outflow by transjugular intrahepatic portosystemic shunt (TIPS) procedure. However, in our patient with complete portal vein occlusion and large PSS, additional and new creation of a portosystemic shunt can cause complications such as hepatic encephalopathy and hepatic failure^[25,26]. We therefore decided to use a transhepatic approach to treat PVT. The transhepatic approach is technically simpler than transjugular approach. This approach allows direct guidewire recanalization of the occluded portion and instillation of high concentrations of UK directly into the thrombus, and reduces the risks of systemic fibrinolysis. In this transhepatic approach there is a risk of uncontrollable bleeding through the transparenchymal tract after removal of the catheter especially in patients with coagulopathy^[27]. Avoiding such complications, complete transparenchymal tract embolization with microcoils or gelatin sponge after removal of the catheter is necessary to overcome intraabdominal bleeding. In our case, the transparenchymal tract was embolized with microcoils, and PVT was dissolved completely by transhepatic catheter-directed thrombolysis without hemorrhagic complications. We believe that local

thrombolytic therapy *via* transhepatic route should be considered as a feasible, efficacious and safe treatment.

In the present case, even after recanalization of the portal vein, the patient had stagnancy of portal blood flow with outflow of the SMV into the large PSS. Unless the blood flow to PSS is decreased to increase the portal blood flow, PVT may recur after dissolution. Concurrent BRTO was therefore performed to occlude the splenorenal shunt in our patient, and achieved long-term patency of the portal vein. There is no previous report describing the necessity for concurrent occlusion of portosystemic shunt after recanalization of PVT.

In conclusion, catheter-directed thrombolysis combined with BRTO is feasible and useful for recanalizing PVT and maintaining stable patency of the portal vein in patients with PVT.

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Clinical evaluation of submucosal colonic lipomas: Decision making

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Abstract

Even lipomas are the most common mesenchymal benign tumors of the gastrointestinal tract, symptomatic colonic presentation is rare. Herein, we evaluated four patients suffering from various size of colonic lipomas and approached by different therapeutic modalities.

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Key words: Colon; Lipoma; Complication; Management

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INTRODUCTION

Lipomas are the most common nonepithelial benign tumors of the gastrointestinal tract. Nevertheless symptoms of a colonic lipoma are rare generally with a silent clinical course. When colonic lipomas achieve a proper size, they have manifestations such as change in bowel habits, rectal bleeding, abdominal pain or more disastrous consequences

like obstruction and intussusception requiring urgent interventions. Herein, we report four patients suffering from various sizes of colonic lipoma who were treated with different therapeutic modalities.

CASE REPORT

The first patient was a 77-year old female who presented with recurrent episodes of right upper quadrant abdominal cramping. Her physical examination and previous medical history were unremarkable except for a cholecystectomy. Standard laboratory values were within normal ranges. Abdominal computed tomography (CT) revealed a regular contoured 4 cm × 3 cm mass lesion with fatty density localized in the midportion of ascending colon causing a luminal narrowing defect (Figure 1A). In double contrast enema, a filling defect of the protruded polypoid lesion was detected at the same location (Figure 1B). Colonoscopy demonstrated a mass lesion with a broad base and normal overlying mucosa (about 4 cm in diameter) adjacent to the hepatic flexura. The patient was diagnosed having a symptomatic colonic lipoma during surgery. Following colotomy, mucosa overlying the mass lesion was dissected and a tumor with a macroscopic appearance similar to lipoma was enucleated (Figure 1C). Histopathologic examination verified the diagnosis of lipoma localized in the submucosal layer and the patient remained asymptomatic through a 2-year follow-up period.

The second patient was a 41-year old male admitted to our clinic with similar complaints of the first patient. Physical and laboratory examinations were natural only with a positive fecal occult blood test. He denied any significant medical or surgical history. Abdominal CT demonstrated a regular contoured 3 cm × 2 cm mass lesion with fatty density localized in the hepatic flexura causing a filling defect which was confirmed by double contrast enema (Figure 2A). A segmental resection was performed with the diagnosis of lipoma, pathological evaluation of the specimen showed that it was a benign submucosal lipoma (Figure 2B).

The third patient was a 77-year old female presented with recurrent abdominal pain in the left lower quadrant for a year, bloody defecation for 6 mo and constipation for 4 wk. Physical examination revealed no pathological change except for left lower quadrant distention. Blood biochemical analysis, sedimentation rate and complete blood count were normal. Colonoscopy revealed a broad-

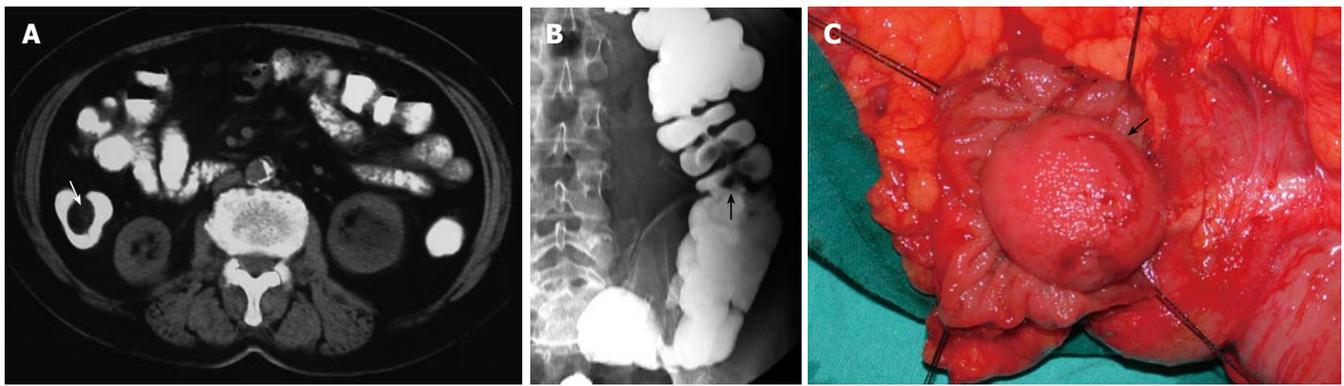


Figure 1 Computed tomography (CT) scan showing a regular contoured 4 cm x 3 cm lesion with fatty density localised in midportion of ascending colon causing a luminal narrowing defect (A), double contrast barium enema showing a filling defect of the protruded polypoid lesion at the hepatic flexura of colon (B), and appearance of the colonic submucosal lipoma during the operation (C).

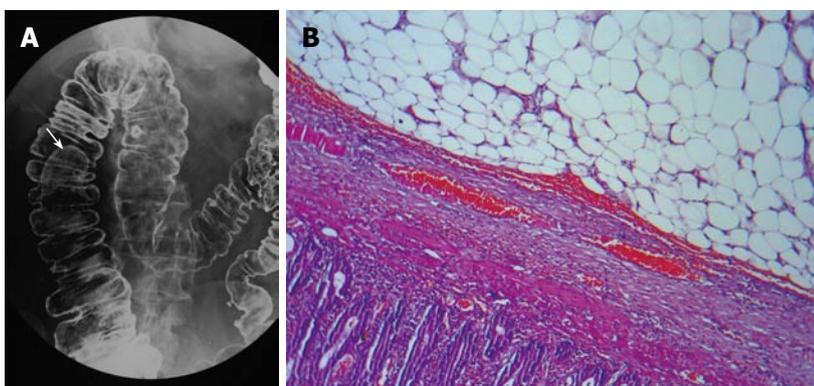


Figure 2 A mass lesion located in the hepatic flexura causing filling defect shown by double contrast enema (A), resected biopsy specimen showing a submucosal benign lipoma composed of mature lipocytes by hematoxylin and eosin staining (B) (x 400).

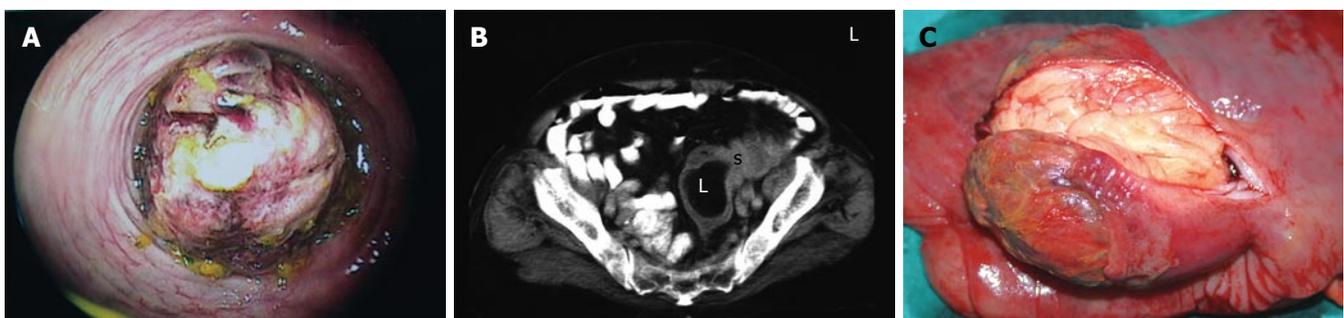


Figure 3 Colonoscopic appearance of a broad-based giant mass acquiring spontaneous hemorrhagic areas and obstructing more than 75% of the lumen of sigmoid colon (A); abdominal computed tomography scans showing the diffuse thickening of sigmoid colon wall, invagination and a distal intraluminal giant mass with fat density with a size of approximately 6 cm x 7 cm (B); macroscopic appearance of the giant sigmoid colon lipoma (C).

based giant mass acquiring spontaneous hemorrhagic areas and obstructing more than 75% of the colonic lumen (Figure 3A). Punch biopsy exhibited benign colonic mucosa showing inflammatory granulation. Abdominal CT showed diffuse thickening of the sigmoid colon wall, invagination and an intraluminal mass with a size of approximately 6 cm x 7 cm having density values equal to fat (Figure 3B). An evident sigmoid redundancy and a mass lesion nearly obliterating colonic lumen were detected during surgery. A segmenter resection of the sigmoid colon was the operative procedure of choice (Figure 3C). Microscopic examination confirmed that the lesion was a submucosal lipoma with a size of 6 cm x 7 cm.

The fourth patient was a 44-year old male presented with tenesmus. Physical examination revealed nothing significant. A pediculated polypoid mass lesion projecting into the lumen with an approximate diameter of 2.5 cm was discovered at the 15th cm of the rectum by colonoscopy. An endoscopic snare polypectomy and rectal biopsy were performed during the same intervention. The histopathologic examination revealed normal rectal mucosa and underlocated lipoma.

DISCUSSION

Lipoma of the gastrointestinal tract was first described by

Bauer *et al*^[1] in 1757. Lipoma is the second most common benign colonic tumor following adenomatous polyps and the incidence has been reported to range between 0.2% and 4.4%^[2,3]. Colonic lipoma is more common in elderly women and tends to derive from right hemicolon with a decreasing frequency from cecum to sigmoid colon^[2-5]. Approximately in 90% of cases, lipoma is defined to arise from the submucosal layer and the subserosal or intermucosal layer accounting for the remaining 10%^[1,6]. They are usually solitary with varying sizes and may be sessile or pedunculated. Although the majority of these lesions are asymptomatic and detected incidentally during the examination of symptoms like abdominal pain, change in bowel habits, and rectal bleeding or in surgical specimen removed for various other reasons, on rare occasions colonic lipoma may present with massive hemorrhage, obstruction, perforation, intussusception, or prolapse^[7-11]. Severity of the signs and symptoms is attributed to the size of the lesions. Lipomas larger than 2 cm in diameter may cause symptoms such as constipation, diarrhea, abdominal pain, or rectal bleeding^[1,3,5]. Colicky pain may be due to intermittent intussusception whereas rectal bleeding can occur as a result of ulceration of the overlying mucosa. One of the greatest clinical significances of lipoma is its potential to be confused with colonic malignancies according to the similarity in both symptomatology, fortunately sarcomatous changes in colonic lipomas have not been reported yet^[1]. Colonoscopy, CT and barium enema are considered to be the diagnostic tools, but physicians should be aware that colonoscopic biopsies usually possess no histopathologic value as the lesion is beneath the normal mucosa and biopsy often can not promote diagnosis^[12]. The radiographic appearance of colonic lipomas may resemble that of carcinomas. The "squeeze sign" is told to be pathognomonic for colonic lipomas, and is characterized by the elongation of a spherical filling defect during peristalsis on barium enema examination^[1,13]. Despite recent diagnostic innovations in radiology, histopathologic evaluation is the gold standard in precise diagnosis. However, most colonic lipomas are asymptomatic incidental entities, the decision of removal is based on the criteria including those with suspicion of malignancy, and symptomatic lipomas. A wide range of treatment modalities have been suggested. The way of removal depends on the presentation of the case as an elective or an emergency. Surgical intervention is mandatory in surgical emergencies such as obstruction, intussusception, perforation, or very rarely massive hemorrhage. Elective endoscopic removal of submucosal lipoma up to 2 cm in diameter is reported to be safe and appropriate, however in larger lesions the risk of associated complications like uncontrolled hemorrhage and perforation makes the procedure controversial^[1,14-16]. Conventional laparotomy including enucleation, colotomy and excision, and segmental colonic resection has been described as a choice of treatment as well as minilaparotomy or transanal resection of lesions mimicking rectal prolapse^[1,7,16]. Minimally invasive surgeries such as laparoscopy-assisted re-

section under colonoscopic guidance has also been defined for selected cases^[15,17].

In the literature, the general consensus on colonic lipomas is that local enucleation is an appropriate and effective procedure of choice for the management of these lesions possessing no malignant potential. Colonoscopic removal can be safely performed for submucosal lipomas smaller than 2.5 cm in diameter, however larger or subserosal tumors associated with complication risks of obstruction, intussusception, hemorrhage and even perforation deserve extended interventions. Surgical approach should be established through the features of each case on individual basis.

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CASE REPORT

Adverse hepatic reactions associated with calcium carbimide and disulfiram therapy: Is there still a role for these drugs?

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Abstract

Disulfiram and calcium carbimide are two alcohol deterrents widely used in alcoholism treatment, however, there exist great concerns over their safety. Reports on hepatotoxicity, mainly related to disulfiram therapy, have been published. The hepatotoxic potential of calcium carbimide is less well characterized. Here, we describe four cases of liver damage related to this therapeutic group that were submitted to a Registry of hepatotoxicity and point out the limitations that we face when prescribing these compounds. A reassessment of the role of these compounds in the management of alcohol dependence is clearly needed.

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Key words: Calcium carbimide; Disulfiram; Hepatotoxicity

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INTRODUCTION

Disulfiram (tetraethylthiuram disulphide), an alcohol deterrent, is widely used in alcoholism treatment. Another

drug, calcium carbimide is less frequently prescribed and not on the market in many European countries, being commercialized in Australia, Canada, South Africa, Austria, Netherlands, Sweden and Spain, among others. These drugs produce physiological changes after ethanol consumption, which are sufficiently unpleasant to dissuade most alcoholics from further drinking. However, there exist great concerns over their safety. Both agents share a similar profile of adverse effects including dermatological and haematological reactions^[1]. Reports on hepatotoxicity, mainly related to disulfiram therapy and leading in some cases to fulminant liver failure, have been described^[2,3]. Indeed, in a recently published study by Björnsson & Ollson (2005) aimed to analyse the outcome of patients with severe drug-induced liver injury, disulfiram stood out as the second most commonly reported drug associated with mortality^[4]. Conversely, the hepatotoxic potential of the less prescribed calcium carbimide is not well recognized. We describe four cases of liver damage related to this therapeutic group that were submitted to a Registry of hepatotoxicity in work since 1994. The operational structure of the Registry, data recording and case ascertainment have been summarily reported elsewhere^[5]. Only cases considered drug-related by experts' clinical judgment were assessed by the Council for International Organizations of Medical Sciences scale (CIOMS scale)^[6], that provides a standardized scoring system according to the type of liver injury and based on six axes of decision-taking.

CASE REPORT

A 23-year-old man (patient 1) was prescribed calcium carbimide (120 mg/d) in July 1997. He was admitted to the hospital in August 1997 because of a week's history of fatigue, dark urine, and pruritus. Physical examination showed a pruriginous exanthema, marked jaundice, without any signs of chronic liver disease although he admitted an alcohol consumption of 80-100 g/d, and laboratory findings at presentation are shown in Table 1. Serology ruled out viral causes, screening for autoantibodies was negative and findings of an abdominal ultrasonographic examination were normal. A liver biopsy showed perivenular cholestasis and mild hepatobiliary damage. Liver tests were normal on 49 d after drug withdrawal. This case yielded 11 points when applying the CIOMS scale which fell into the category of highly probable.

A 39-year-old man (patient 2) on chronic alcohol con-

Table 1 Serum concentrations at time of presentation with calcium carbimide or disulfiram induced liver damage

Patient	Bilirubin(mg/L)		ALT(U/L)	AP (× ULN)	Type of liver injury
	Total	Direct			
1	22.4	14.4	553	× 3.4	Cholestatic hepatitis ¹
2	0.9	0.2	59	× 0.56	PAS-positive ground glass hepatitis ¹
3	11.8	8.43	713	× 1.4	Hepatocellular
4	27.1	16.72	1288	× 1.49	Septal fibrosis and active Chronic hepatitis ¹

¹ Liver biopsy findings. Abbreviations: ALT, alanine aminotransferase ($n < 40$ U/L); AP, alkaline phosphatase; ULN, upper limit of normality; total bilirubin ($n < 1.2$ mg/dL). The ALT and AP values are these at presentation where as bilirubin values are the highest recorded.

sumption (110 g/d) was prescribed calcium carbimide (75 mg/d) for aversion therapy in February 1995. After 25 wk of treatment he was admitted to hospital because of an increase in serum transaminases, with a ratio of alanine aspartate and alanine aminotransferase of 1.05 (62/59, UL) (Table 1). The patient admitted alcohol abstinence for the previous 6 mo and during the course of the medical treatment. Physical examination disclosed hepatomegaly without any signs of chronic liver disease. Serology tests ruled out viral causes. Screening for autoantibodies produced negative results, and the results of an abdominal ultrasonographic examination were normal. A liver biopsy elicited ground-glass inclusion bodies in the hepatocytes associated with portal inflammation and fibrosis. The patient did not attend any further appointments.

A 42-year-old woman (patient 3) was prescribed venlafaxine (75 mg/d) and alprazolam (1.5 mg/d) for depression, and calcium carbimide (75 mg/d) since December 2002. Mean daily alcohol ingestion was 120 g/d that was stopped 45 d prior to admission. The results of prior liver function tests were normal. After 75 d of treatment she was admitted to hospital because of asthenia and fever, and a diagnosis of spontaneous bacterial peritonitis was made. Physical examination showed a jaundiced patient without stigmata of chronic liver disease. Serum chemistry indicated hepatocellular injury (Table 1) without evidence of viral causes. Over the next days she developed fulminant hepatic failure unresponsive to molecular adsorbent recirculating system (MARS) therapy which was unsuccessfully instituted three times and died on d 31 after hospitalization. This case yielded 6 points when applying the CIOMS scales which fell into the category of probable.

A 48-year-old man (patient 4) was admitted to hospital in August 2003 because of a five-day history of fatigue and dark urine one month after automedication with disulfiram (1000 mg/d). He had been taking phenytoin 200 mg/d for epilepsy for 5 years. He was a heavy alcohol drinker (> 80 g/d) and although he denied alcohol intake in the previous month he was actually an active consumer. Physical examination showed marked jaundice with signs of chronic liver disease. Serum chemistry indicated hepatocellular injury (Table 1). Screening for viral disease was negative and abdominal ultrasonographic examination

showed normal biliary ducts and moderate ascites. Liver biopsy revealed a chronic active hepatitis and septal fibrosis. Laboratory findings returned to previous baseline values 9 mo after drug withdrawal.

We described four cases of adverse liver reactions related to the use of calcium carbimide (3 cases) and disulfiram (1 case) because there was a temporal relationship between the administration of the drugs and the onset of hepatic abnormalities as well as between withdrawal of the drugs and improvement in liver dysfunction, the only exception being patient 3 who developed fulminant liver failure. In patient 2 de-challenge could not be ascertained because the patient was lost to follow up. Indeed, in this patient the increase in alanine aminotransferase was less than two times the upper limit of normal and since the biochemical expression of liver injury according to the International consensus criteria^[7] was a biological damage, causality assessment with the CIOMS scale was not performed. However, he presented in the liver specimen the hepatocyte inclusions seen only with calcium carbimide therapy along with fibrosis and lymphocyte portal inflammation characteristic of a chronic form of liver damage^[2,8].

On the other hand, causes of liver damage (viral, immunologic and metabolic) were ruled out. In patient 4, liver biopsy ruled out a diagnosis of alcoholic hepatitis. Patient 3 was prescribed venlafaxine which has been associated with non fatal cases of hepatotoxicity^[9].

The mechanism of aversion therapy-induced hepatotoxicity is presumed to be idiosyncratic. The absence of clinical features of hypersensitivity in these cases suggests that a toxic metabolite may be responsible, although an immune mechanism has been implicated in some cases^[3]. Both compounds are extensively metabolized in the liver and block the oxidation of alcohol at acetaldehyde stage increasing acetaldehyde blood levels. Therefore, their administration requires the agreement of the patient to stop drinking. In our series patient 4 self administered disulfiram while continuing drinking which could have rendered him at risk of severe medical complications. Furthermore, another concern related to the use of alcohol deterrents is the possibility of drug interactions. Actually, they may inhibit the metabolism of drugs such as phenytoin (CYP 2C19) and an interaction with the antidepressant venlafaxine cannot be discarded. Coadministration of aversive therapy with other drugs (antibiotics, amitriptyline, *etc.*) could aggravate/precipitate the disulfiram-alcohol reaction. It is not yet known whether associate medications might contribute to the hepatotoxic effect of these drugs^[1]. It is also important to highlight that since these compounds may be prescribed to patients with underlying alcoholic liver disease such as liver cirrhosis and alcoholic hepatitis (although they should be cautiously prescribed if at all in this population), the diagnosis of hepatotoxicity may go unrecognized.

In summary, these cases exemplify the main limitations of use that these two principal alcohol-sensitizing drugs present. Furthermore, since the efficacy of aversive therapy remains to be proved^[10], a reassessment of the role of these compounds in the management of alcohol dependence is clearly needed.

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Unusual prerectal location of a tailgut cyst: A case report

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Abstract

Tailgut cyst is a rare congenital cystic lesion arising from the remnants of the embryonic postanal gut. It occurs exclusively within the retrorectal space and rarely in the perirenal area or in the subcutaneous tissue. A prerectal and retrovesical location of tailgut cyst is extremely rare. To the best of our knowledge, only three cases have been reported in the English literature. We experienced an unusual case of tailgut cyst developed in the prerectal and retrovesical space in a 14-year-old boy. Abdominal computed tomography demonstrated a prerectal cyst which was located at the anterolateral portion to the rectum. The cyst contained yellowish inspissated mucoid material. Microscopically, the cyst was lined by squamous, columnar, cuboidal and transitional epithelia and the wall was fibrotic with dispersed smooth muscle cells. Although tailgut cyst arising in prerectal area is extremely rare, its possibility should be considered in differential diagnosis of a prerectal and retrovesical cystic mass.

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Key words: Tailgut cyst; Prerectal mass; Retrovesical mass; Developmental cyst

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INTRODUCTION

Tailgut cyst, known as a retrorectal cystic hamartoma, is a rare congenital multicystic lesion arising from the remnants

of the embryonic postanal gut^[1-3]. It almost exclusively occurs within the retrorectal or presacral space^[4] and rarely in the perirenal area^[2,3,5], the subcutaneous tissue in the anorectal region^[6-9] and anterior to the rectum^[4,10]. The prerectal location of a tailgut cyst is extremely rare. To the best of our knowledge, only three cases have been reported in the English literature. Here we report an additional case of tailgut cyst arising in the prerectal and retrovesical space and review the relevant literature.

CASE REPORT

A 14-year-old boy presented with abdominal pain and dysuria for 2 d. Physical examination revealed suprapubic pain and tenderness. Routine laboratory tests including a complete blood count, electrolytes, and routine urine analysis were within normal limits. Abdominal computerized tomography demonstrated a well-demarcated oval-shaped cystic mass which was located at the prerectal and retrovesical space (Figure 1). He underwent explorative laparotomy for the definite diagnosis and treatment. Intra-operative finding showed that the cyst did not communicate with the prostate, bladder or rectum. Complete mass excision was performed. Macroscopic examination revealed a unilocular cyst measured 5 cm × 3 cm in cross diameter. The cyst showed a thickened fibrous wall and focal adhesion to the adjacent fibroadipose tissue. The cyst contained yellowish inspissated mucoid material. Microscopically, the cyst showed a variety of the lining epithelia including glandular, cuboidal, transitional and squamous epithelia (Figure 2). The cyst wall contained disorganized bundles of smooth muscle cells. The underlying stroma showed mild infiltration of chronic inflammatory cells. On immunohistochemical staining, prostate specific antigen (PSA) and neuroendocrine markers including chromogranin A were negative in the lining of the epithelial cells. The patient's recovery remained uneventful for 10 mo postoperation.

DISCUSSION

Tailgut cyst or retrorectal hamartoma, is a rare congenital multicystic lesion and is believed to originate from the remnant of the tailgut which is a primitive gut temporarily present at the caudal portion of the embryo^[2,3,8]. During its development the embryo possesses a true tail, reaching its largest diameter on the 35th d of gestation. The anus develops above the tail on the 56th d of gestation, by which time the latter has completely regressed. Remnants of the tailgut or neuroenteric cord may be the origin of tailgut

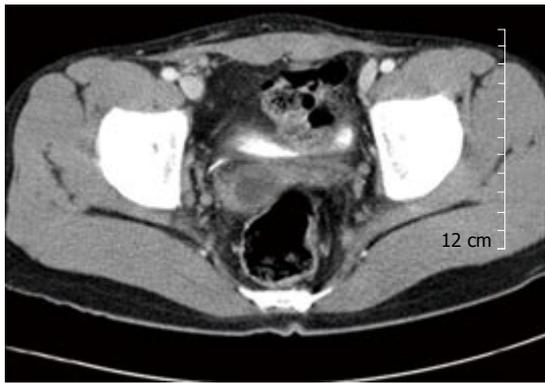


Figure 1 Abdominal computed tomography revealing an oval-shaped cyst with a thickened wall in anterolateral to the rectum.

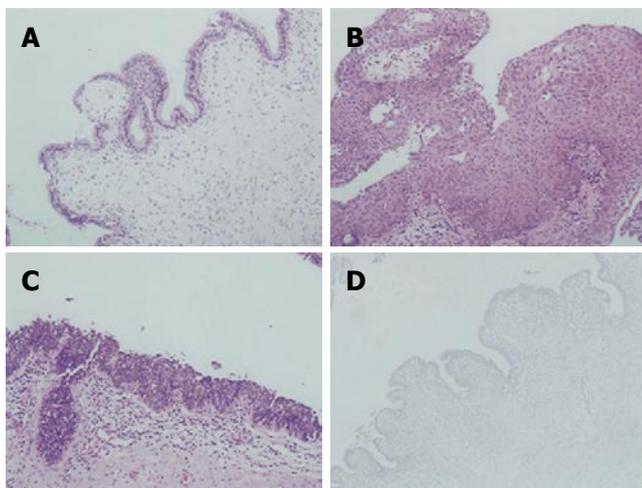


Figure 2 Microscopically, the cyst wall showing a variety of lining epithelia including tall columnar or cuboidal (A), squamous (B), and transitional (C) epithelium (HE, x 200), the underlying stroma showing a mild infiltrate of chronic inflammatory cells, immunohistochemical staining showing negative prostate specific antigen in the lining of epithelial cells (D) (PAP, x 200).

cysts^[3,11].

Tailgut cyst can affect at any age but predominantly middle aged women. The female to male ratio is about 3:1. Half of them are asymptomatic and are frequently found on routine physical examination^[1,4]. Symptomatic patients present with abdominal pain, rectal bleeding, and rectal fullness^[2]. Complete surgical excision is necessary for accurate diagnosis and treatment, and prevents complications of the tailgut cyst, including infection, recurrence, and malignant transformation^[1]. Tailgut cyst is usually a well-circumscribed, soft, and multicystic or multilocated mass with adherent surrounding fibroadipose tissue. The varying-sized cysts contain clear serous or translucent mucoid fluids. Microscopically, the cysts are lined by a wide variety of epithelia varying from cyst to cyst, or even within the same cyst. Epithelia can be divided into the following types, such as the stratified squamous type, transitional and cuboidal type, stratified columnar type, ciliated pseudostratified columnar and gastric type. In most cases the cyst wall contains well-formed disorganized focal bundles of bland smooth muscle cells^[2,3].

Tailgut cyst occurs almost exclusively in the retrorectal

or presacral space. Infrequently, the cysts extend from the presacral space to involve the rectal wall and rarely extend laterally from the mid-line or into the postsacral space or lateral and anterior to the rectum^[4], but occur rarely in the perirenal area, the subcutaneous tissue in the anorectal region and anterior to the rectum^[2-10]. The prerectal location of tailgut cyst was noted in our patient. To the best of our knowledge, only three cases have been reported in the English literature^[4,10].

The main differential diagnoses of the prerectal tailgut cyst include the utricle cyst in the prostate, the rectal duplication and the simple cyst in the seminal vesicle. The prostatic utricle cyst, known as mullerian duct remnant, is a midline cyst of the prostate and also occurs in the prerectal or retrovesical area. Histologically, the cyst is lined with stratified columnar or cuboidal epithelium and continuous with the utricular wall. Immunohistochemically, the cyst lining epithelial cells show immunoreactivity for PSA and the neuroendocrine cells show immunoreactivity for chromogranin A^[12]. Duplication cyst is lined by epithelium similar to that of the gastrointestinal and respiratory tracts. The main distinctive feature is a well-formed muscular wall overlying two layers of muscular bundles containing a nerve plexus^[2,3]. The simple cyst in the seminal vesicle is an unilocular cystic lesion located at lateral to the midline in the retrovesical area. The cyst is lined with a cuboidal or flattened epithelium with a fibrous wall of variable thickness. Characteristically, the fluid within the cyst contains sperm or sperm fragments^[13].

In conclusion, we report an unusual case of tailgut cyst arising in prerectal and retrovesical area in a 14-year-old boy. Although tailgut cyst arising in prerectal area is extremely rare, its possibility should be considered in differential diagnosis of a prerectal and retrovesical cystic mass.

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CASE REPORT

Cytomegalovirus ileitis in an immunocompetent elderly adult

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Abstract

Cytomegalovirus enteritis is most usually associated with patients positive for human immunodeficiency virus or immunosuppressed transplant patients. The gastrointestinal tract may be affected anywhere from the esophagus to the colon, but the small bowel involvement is rare. We report a case of cytomegalovirus ileitis in an immunocompetent adult, which was confirmed by histopathologic findings through colonoscopic biopsy.

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Key words: Cytomegalovirus; Ileitis; Immunocompetent

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INTRODUCTION

Cytomegalovirus (CMV) is a β -group herpes virus and produces latent infection like other herpes viruses. Clinically significant CMV infection usually occurs in patients with immunosuppression, transplantations and acquired immune deficiency syndrome^[1]. In immunocompetent subjects, active infection is apparently rare, and the virus exhibits relatively low pathogenicity^[2]. We here describe a case of CMV ileitis without colon involvement in an immunocompetent subject.

CASE REPORT

A 63-year-old woman, with a one-day history of epigastric pain, was hospitalized at Ewha Womans University Hospital on Feb 16, 2006. She is a non-smoker and denied any medication or drug abuse.

On physical examination, the patient appeared to be acutely ill, with a blood pressure of 130/80 mmHg, heart

rate of 70 beats/min, respiratory rate of 20 breaths/min, and temperature of 37°C. The abdominal examination was notable for the presence of pain on the epigastric and the right lower quadrant areas with mild guarding but bowel sounds were normal. Laboratory values on admission were in normal range except elevated C-reactive protein.

The chest X-ray was normal. Abdominal computed tomography showed layering appearance of bowel wall and luminal dilatation from the distal jejunum to the pelvic ileal loop (Figure 1). The colonoscopy revealed scattered regions of geographic deep ulcerations and hyperemic edematous mucosa upon a background of normal mucosa in terminal ileum (Figure 2). We made differential diagnosis including nonspecific ileitis, Crohn's disease, tuberculosis induced ileitis and ischemic ileitis. Histological examination showed mild inflammation with lymphocytic infiltration without specific feature.

After the initial clinical and laboratory evaluation, the patient began therapy with intravenous antibiotics and fluids for the most probable diagnosis of nonspecific ileitis. During the admission, her clinical condition improved, and abdominal pain disappeared. Two weeks later, the follow up colonoscopy was performed. The ulcerations and hyperemic edematous lesions, which were previously noted on the terminal ileum, were much improved (Figure 3). The histological findings showed intranuclear and intracytoplasmic inclusions. Immunohistochemical stain for CMV showed scattered positive cells at the ulcer base (Figure 4). She was discharged without medication on March 7, 2006.

DISCUSSION

CMV is a member of the herpes virus family and is a prevalent pathogen, with 40 to 90 percent of the general population showing prior exposure by serology^[3]. Human CMV infection can be seen at all stages of life but most often affects those who are immunosuppressed and can commonly cause retinitis, pneumonitis or enteritis^[4]. Most primary CMV infections in immunologically healthy adults do not cause symptoms or are associated with a mild mononucleosis like syndrome^[5].

Gastrointestinal involvement in CMV infection is well known among immunocompromised patients. It is usually seen in advanced acquired immune deficiency syndrome with CD4 lymphocyte count less than 50 cells/mm³ and among transplant recipients as a reactivation of latent infection or rarely during primary infection^[6]. CMV colitis in immunocompetent individuals has been reported mainly in older patients, particularly those with other



Figure 1 Abdominal computed tomography on admission revealed layering appearance of bowel wall and luminal dilatation from the distal jejunum to the pelvic ileal loop.



Figure 3 Follow up colonoscopy was performed. The previously noted ulcers and hyperemic edematous lesion were improved.



Figure 2 Colonoscopy, performed on admission, revealed scattered regions of geographic ulcers and hyperemic edematous mucosa on the terminal ileum.

comorbidities, and is mostly community-acquired. This is likely a reflection of a weakened immune system, as a consequence of aging. Age-related dysfunctions of B and T cell lymphocytes, impaired cytokine, and perturbation of mucosal immunity are thought to contribute to a relative immunodeficiency in the elderly, predisposing them to various infectious and inflammatory diseases^[7].

Gastrointestinal tract may be affected anywhere from the mouth to the anus with the esophagus and colon being most common and small bowel relatively rare. The clinical manifestations of gastrointestinal CMV infection include gastrointestinal bleeding, abdominal pain, vomiting and diarrhea^[1,8]. Endoscopic findings of gastrointestinal CMV disease include erosions, ulceration and mucosal hemorrhage. The prototypical ulceration has a well-defined punched out appearance. The pathogenesis of CMV-induced ulceration is thought to involve ischemic mucosal injury secondary to infection of the vascular endothelial cells^[8,9].

CMV enteritis is remarkable for its ability to mimic many diseases, such as colitis caused by *C. difficile*, ischemia, malignant diseases of the colon and inflammatory bowel diseases. The diagnosis is made by the demonstration of the presence of CMV by routine histologic examination, culture or staining for CMV antigen or DNA^[10,11].

The primary therapy for clinically significant CMV

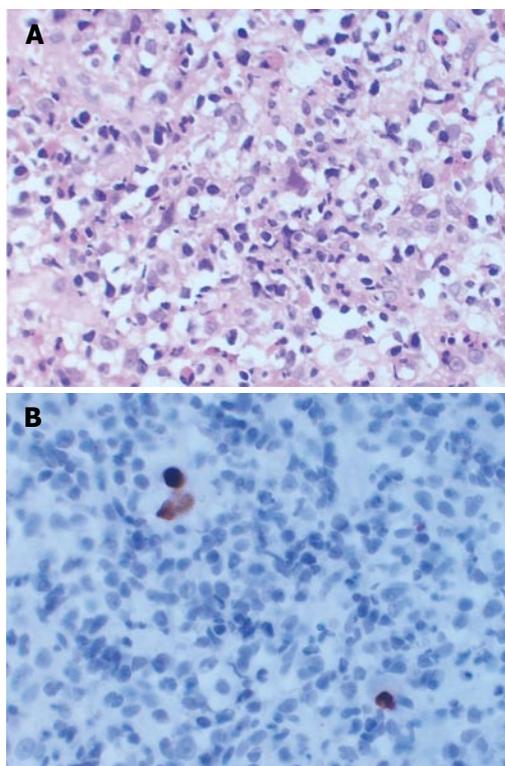


Figure 4 Pathologic findings. **A:** Enlargement of infected cells and intranuclear inclusions were noted (HE stain, $\times 100$); **B:** Immunohistochemical stain for CMV showed scattered positive cells.

infection is use of the antiviral drugs such as ganciclovir and foscarnet^[6,12]. Galiatsatos *et al*^[7] reported that spontaneous resolution occurred mainly in patients younger than 55 years of age, with no other comorbidities. They suggested that young, otherwise healthy, well-selected patients can improve without the need for antiviral therapy but immunocompromised patients and patients older than 55 years of age with comorbidities need antiviral therapy. Sakamoto *et al*^[13] thought that the administration of the antiviral agents is not always necessary for immunocompetent individuals.

In conclusion, we report a case of CMV ileitis not involving colon in an immunocompetent elderly adult who recovered without antiviral therapy.

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Pseudoachalasia in a patient after truncal vagotomy surgery successfully treated by subsequent pneumatic dilations

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Abstract

Pseudoachalasia is a difficult condition for the clinician to differentiate from idiopathic achalasia even by manometry, radiological studies or endoscopy. Its etiology is usually associated with tumors. In most cases, the diagnosis is made after surgical explorations. The proposed pathogenesis of the disease is considered as mechanical obstruction of the distal esophagus or infiltration of the malignancy that affects the inhibitory neurons of the myenteric plexus in the majority of cases. Surgery has been reported as a cause of pseudoachalasia. We report a 70-year-old man who suffered from deglutination disorder caused by pseudo-achalasia after truncal vagotomy. The patient was symptom-free after a nine-year follow-up and complete recovery of esophageal motility status from pseudoachalasia after pneumatic dilations. We also reviewed the literature of pseudoachalasia.

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Key words: Truncal vagotomy; Pseudo-achalasia; Deglutination disorder; Pneumatic dilations; Sustain reversed esophageal motility

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INTRODUCTION

Pseudoachalasia is a disease entity usually associated with tumors causing obstruction at the gastroesophageal junction and producing abnormal esophageal motility mimicking an idiopathic achalasia^[1]. Idiopathic achalasia is a motility disorder of the esophagus characterized by aperistalsis and failure of the lower esophageal relaxation, resulting in symptoms such as dysphagia, regurgitation, chest pain and weight loss. The pathogenesis of idiopathic achalasia is due to the selective loss of inhibitory neuron of the myenteric plexus which produces vasoactive intestinal polypeptide (VIP), nitric oxide and inflammatory infiltration and is responsible for the unopposed excitation of lower esophageal sphincter (LES)^[2-4], thereby causing dysfunction or failure of the LES relaxation in response to each swallow^[4,5]. The pathogenesis of pseudoachalasia is different from idiopathic achalasia. In pseudoachalasia, the proposed pathogenesis is considered as a mechanical obstruction of the distal esophagus or an infiltration of the malignancy that affects the inhibitory neurons of the myenteric plexus. Endoscopy and radiologic tests are often important in distinguishing between them, although the eventual diagnosis is made only by surgical explorations in the most cases. These both types of achalasia share the same manometric phenomenon of aperistalsis of the esophageal body and incomplete LES relaxation. However, one report suggested that patients with achalasia have autonomic nerve dysfunction in the vagal nerve outside the esophagus^[6]. Trauma to the gastroesophageal junction may potentially damage the vagus nerve, thereby leading to secondary achalasia. Most case reports on pseudoachalasia were tumor related, but implicated vagal nerve injury following surgery as a cause of achalasia^[8-10] still occurred. We report herein a reversible pseudoachalasia following the truncal vagotomy for pyloric obstruction, which was successfully treated by pneumatic dilations.

CASE REPORT

A 70-year-old male, suffered from repeated peptic ulcer bleeding for at least 10 years, developed progressive symptoms of post-prandial vomiting in October 1996.

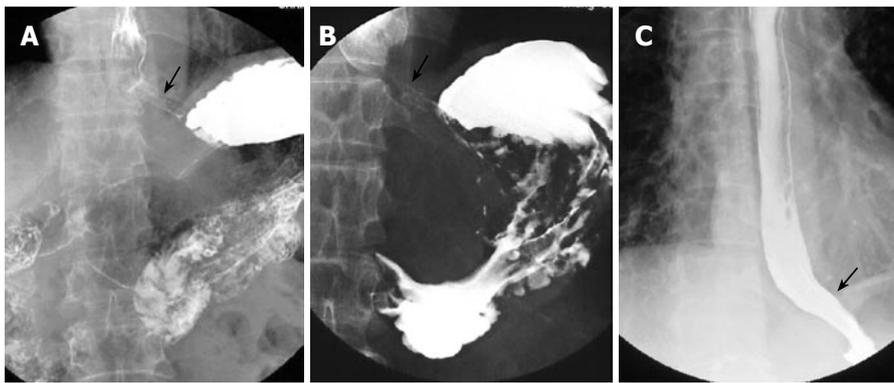


Figure 1 A: Barium esophagography and upper gastrointestinal series showing an intact gastroesophageal junction; B: dilated distal esophageal lumen with food retention and narrowing of gastroesophageal junction after truncal vagotomy; C: barium esophagography showing a non-dilated esophageal lumen with smooth passage of contrast medium through gastroesophageal junction after pneumodilations.

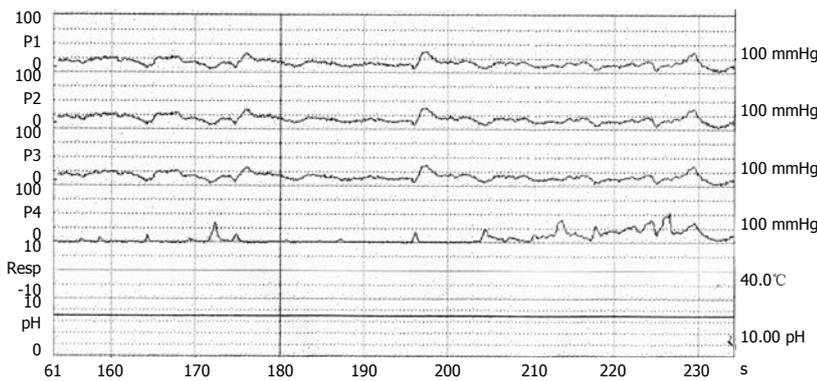


Figure 2 Confirmation of achalasia by manometric findings of esophageal aperistalsis and incomplete relaxation of the lower esophageal sphincter during wet swallowing.

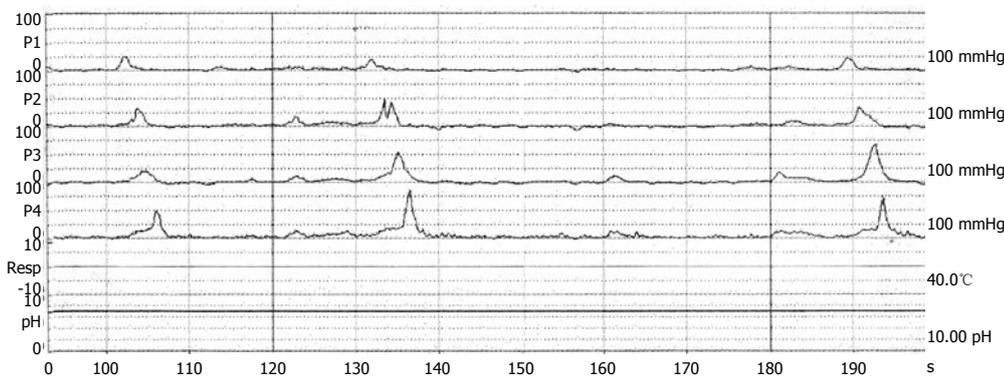


Figure 3 Manometric studies showing restoration of esophageal motility with normal peristalsis as a healthy subject after dilations.

A barium esophagogram and upper gastrointestinal series demonstrated an intact gastroesophageal junction (Figure 1A) and good esophageal peristalsis, but deformed gastric antrum and a deep ulceration. Malignancy was excluded by endoscopy biopsy. Under the condition of pyloric obstruction, he received a truncal vagotomy and pyloroplasty surgery. The month following surgery, the patient developed dysphagia, regurgitation of food. Endoscopy revealed food retention in the esophageal lumen and a tight gastroesophageal junction, thereby suggesting an achalasia. Barium esophagography showed a dilated distal esophageal lumen with food retention and narrowing of gastroesophageal junction (Figure 1B). Achalasia was confirmed using manometric findings of esophageal aperistalsis and incomplete relaxation of the lower esophageal sphincter during wet swallowing (Figure 2). Although the residual pressure was about 12 mmHg but the basal LES pressure was only about 25 mmHg. Achalasia caused by tumors was excluded by

computed tomography and endoscopic ultrasonography. The patient received two episodes of pneumatic dilatation. The symptoms gradually resolved within 4 mo after dilatation and the manometric studies showed restoration of esophageal peristalsis and normal swallowing relaxation (Figure 3). Barium esophagography showed a non-dilated esophageal lumen with smooth passage of contrast medium through gastroesophageal junction (Figure 1C). After nine years, the patient still remains symptom-free with a normal esophageal motility studies.

DISCUSSION

Pseudoachalasia or secondary achalasia is a motility disorder of the esophagus, and usually cannot be distinguished from primary achalasia^[1]. Approximately 2-4% of achalasia patients who fulfill the manometric criteria may develop in a malignant disease^[1]. The clinical spectrum of pseudoachalasia is almost the same

as idiopathic achalasia; both have progressive dysphagia, retrosternal pain, food regurgitation and weight loss. While the radiological findings show a dilated esophagus with "beak-like" distal end, there is aperistalsis of the esophageal body with an incomplete or non-relaxing lower esophageal sphincter on manometry studies in both groups of patients. Therefore, it is difficult for the clinician to distinguish pseudoachalasia from idiopathic achalasia. Pseudoachalasia has many etiologies, including tumor. Most of the patients with pseudoachalasia reported in the literature suffered from a primary or metastatic malignancy around the esophagogastric junction. Some are from benign diseases, such as pancreatic pseudocysts, mesenchymal tumors of the esophagus and mediastinal fibrosis^[11-15]. In rare instances, pseudoachalasia may be a complication of various surgeries, the majority being anti-reflux procedures, gastric banding and gastrectomy^[9,16,17]. Our case resulted as a complication from truncal vagotomy for pyloric obstruction.

The mechanisms of esophageal motor dysfunction in pseudoachalasia have been proposed as follows^[17]: (1) circumferential obstruction of the lower esophagus and/or LES; (2) infiltration of inhibitory neurons of the esophageal myenteric plexus by tumor cells; (3) neuronal degeneration distant from the primary tumor site with reduction in ganglion cells in the dorsal nucleus of the vagal nerve or in the vagal nerve itself; and (4) an interaction of tumor factors with the esophageal neuronal plexus without a direct infiltration of the esophagogastric junction (paraneoplastic).

Achalasia has been described following fundoplication and was attributed to vagal nerve damage during surgery^[18]. In patients who are found to have an achalasia-type motor dysfunction following anti-reflux surgery, Poulin *et al*^[7] suggested three additional explanations. In type 1, primary achalasia had been misdiagnosed, and dysphagia became obvious only after surgery. An alternative explanation for this type of achalasia may be the assumption of Spechler *et al*^[19] that achalasia may occasionally develop from underlying gastroesophageal reflux disease. Type 2 may be due to extensive development of scar tissue and/or an overly tight fundic wrap. In most instances, the motor abnormality disappears following dilation therapy or reconstruction of the fundoplication, as in our patient. Finally, in type 3, achalasia follows anti-reflux surgery after a more prolonged interval. In contrast to type 2, there is no stenosis or fibrosis of the esophagus or the periesophageal tissue, and myotomy is always an effective therapeutic strategy.

Shah *et al*^[20] reported that 25% of patients diagnosed with achalasia had a history of operative or non-operative trauma to the chest and/or upper gastrointestinal tract, compared with 9.5% of patients with dysphagia and normal manometry, thereby suggesting that, in some cases, operative and non-operative trauma may result in vagal disruption and lead to neuropathic dysfunction with the resultant development of achalasia^[20]. The mechanism of damage may not be the same as vagal transection. Vagal efferent fibers, with their cell bodies in the dorsal motor nucleus of the vagus nerve, have a crucial role in initiating and modulating esophageal peristalsis and lower

esophageal sphincter relaxation. Pathological analyses of operative and postmortem specimens from patients with achalasia have demonstrated abnormalities of both extrinsic and intrinsic innervations of the esophagus and LES. The majority of studies revealed a loss of ganglion cells from the dorsal motor nucleus of the vagus and demyelination of the vagal afferent fibers suggesting denervation^[21,22].

Neural control of the LES includes cholinergic and noncholinergic (or nonadrenergic) inhibitory pathways involving the presentation of nitric oxide, acetylcholine, and VIP. Nitric oxide plays an important role in mediating LES relaxation. Nitrergic neurons are present in the dorsal motor nucleus of the vagus, as the source of preganglionic motor neurons to the LES^[23]. Experimental inhibition of nitric oxide may produce a picture mimicking achalasia on manometry. An alternative explanation for post-traumatic achalasia may involve nitrergic pathways. By some mechanisms, trauma may impair the generation of nitric oxide, resulting in decreased inhibition of the lower esophageal sphincter and an abnormality in sequential relaxation of the body of the esophagus^[24].

Pneumatic dilation provides a safe, effective and relatively inexpensive option for achalasia treatment^[25,26]. The major adverse event caused by pneumatic dilations is esophageal perforation, with a 2%-6% cumulative rate^[25-27]. Less common complications are intramural hematoma, diverticula at gastric cardia, mucosal tears, reflux esophagitis, prolonged post-procedure chest pain, hematemesis without change in hematocrit, fever and angina^[25,26,28]. Pneumatic dilation for a malignancy-induced pseudoachalasia stenosis not only provides an ineffective treatment, but also delays adequate therapy of the underlying disorder. Surgery is the best treatment modality but the use of metallic stents is another therapeutic option for the unresectable ones^[29]. However, pneumatic dilation may improve the pseudoachalasia caused by benign etiology and may provide a complete recovery from stenosis as in our patient.

In summary, implicated vagal nerve injury following surgery can cause pseudoachalasia. The motor normality usually resolved following a therapeutic procedure, such as pneumatic dilation on the gastroesophageal junction. Further studies on comparing esophageal manometry and vagal integrity before and after surgery are required.

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Diabetes mellitus with hepatic infarction: A case report with literature review

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Abstract

Hepatic infarction rarely occurs due to the double supply of arterial and portal inflow. A 53-year-old man with diabetes mellitus developed multiple hepatic infarctions after an episode of fever and diarrhea. The infarction was documented by pathology after partial liver resection. Several causes of hepatic infarction may present in this patient: dehydration and hypotension caused by fever and diarrhea, type 2 diabetes and administration of glibenclamide, diabetic ketoacidosis and widespread atherosclerosis. We suggest that diabetic patient with elevated liver enzyme should be considered the possibility of hepatic infarction.

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Key words: Diabetes mellitus; Hepatic infarction; Computerized tomography

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INTRODUCTION

Hepatic infarction is a rare event because of the dual blood supply of the portal vein and hepatic artery, and extensive hepatic arterial collateral system. It is characterized by parenchymal necrosis involving at least two entire lobules, accompanied by a local circulation insufficiency^[1].

Case reports of hepatic infarction in diabetes mellitus (DM) involved ketoacidosis^[2] and nephrotic syndrome^[3], in which upper abdominal pain was developed. We report a case of multiple hepatic infarction in diabetic patient, who

was misdiagnosed with hepatic carcinoma.

CASE REPORT

A 53-year-old man was admitted to the hospital because of fever complicated with diarrhea for 4 d. There was a 4-year history of diabetes mellitus without proper control, although he was treated with glibenclamide 2.5 mg bid. His past medical history included hypertension for 2 years with the highest blood pressure of 150/100 mmHg. The patient did not complain of upper abdominal pain.

At first, he was admitted to the emergency ward. Blood routine examinations showed: hemoglobin 142 g/L, red blood count $4.58 \times 10^{12}/L$, white blood count $11.7 \times 10^9/L$ with neutrophils 93.1%, platelet count $71 \times 10^9/L$, mean corpuscular hemoglobin concentration 402 g/L; urine tests: ketone (+), protein (\pm), glucose (++++); stool routine examination: white blood cell 2-4/HP. He was treated with antibiotics because of suspicion of intestinal infection. After his condition became relative stable, he was transferred to the general ward.

On admission, physical examination revealed no abnormalities except for a slight fever (37.3°C). A second blood routine examination revealed: hemoglobin 144 g/L, white blood count $8.4 \times 10^9/L$, platelet count $77 \times 10^9/L$; urine routine examination: ketone and protein were negative, glucose (++++); stool routine examination: white blood cell 2-4/HP; liver and renal function tests and blood lipid were normal; blood glucose 13.9 mmol/L; blood potassium 3.1 mmol/L, blood sodium 127.8 mmol/L, blood chlorine 98.9 mmol/L, CO₂ 19.7 mmol/L; coagulation function was normal. The chest X-ray and electrocardiogram showed normal.

Treatment was instituted with insulin, antibiotics, and other drugs to correct microcirculation and electrolyte disturbance. After admission into the general ward for 3 d, the patient had a normal temperature and stool. But abnormalities of liver function tests were found: aspartate aminotransferase 50 U/L (normal value, < 40 U/L) and alanine aminotransferase 65 U/L (normal value, < 40 U/L). Doppler ultrasound disclosed an enhanced echo in the liver, suggesting angioma or carcinoma. Liver computerized tomography showed an abnormal signal in the right lobe (Figure 1). On the ninth day, right hepatic wedge resection was performed and the pathologic diagnosis was multiple hepatic infarction with infection (Figure 2). One month after the operation, the patient had a good recovery.

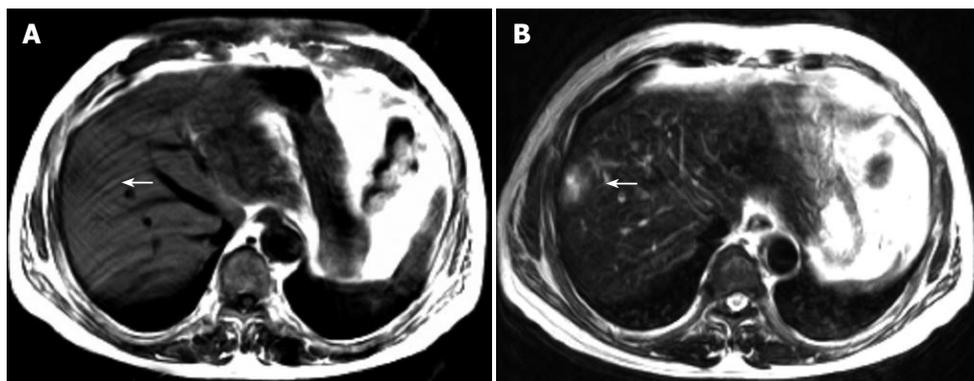


Figure 1 A: Between the V and VIII segments, the right hepatic lobe near the surface has an abnormal signal which seems ellipse. In T₁WI, its center shows long T₁ and slight long T₁ intensity in row; B: In T₂WI, its center shows long T₂ and slightly long T₂ intensity in a row. It shows slight mass effect.

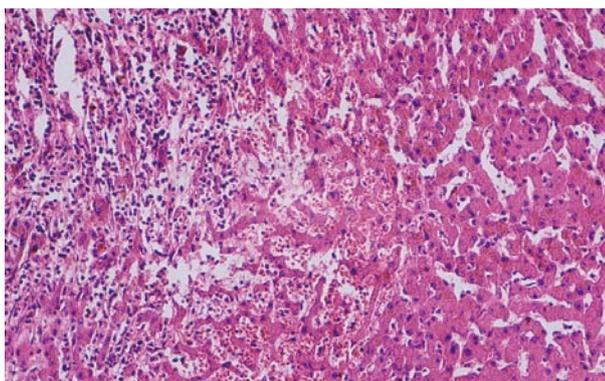


Figure 2 Pathologic section shows hepatic cell necrosis. Vasodilatation and infiltration of inflammatory cells are seen at the junction of necrosis and normal tissues (HE × 100).

DISCUSSION

The hepatic artery supplies about 35% of hepatic blood flow and 50% of the oxygen required by the liver, while the portal vein supplies the remainder^[3]. In addition, an extensive collateral system with 26 possible collateral arteries, and the hepatic arteries do not appear to be end arteries. These factors may explain why hepatic infarction is rare and usually requires impairment of both hepatic arterial and portal venous systems.

Hepatic infarction usually occurs when the hepatic artery or its branches are occluded or when the portal vein is thrombosed. Common cause of hepatic infarction is portal venous thrombosis, and hepatic infarction induced by isolated hepatic artery thrombosis is rare. Apart from vascular occlusion, other factors such as shock, sepsis, anaesthesia, biliary disease and diabetic ketoacidosis may result in hepatic infarction. Surgical factors, including abdominal surgery, laparoscopic surgery, liver transplantation, intra-arterial invasive procedures, transjugular intrahepatic portosystemic shunt, may be common causes of hepatic infarction in clinic^[3,4].

The mechanism of hepatic infarction in this case is unclear. The following factors may contribute to infarction: (1) An episode of fever and diarrhea should be considered to induce dehydration and hypotension, which further decreased both portal and hepatic arterial inflows. (2) Elevated level of catecholamine in diabetic ketoacidosis

might induce vasoconstriction causing ischemia and infarction^[2]. (3) Oral antidiabetic agent glibenclamide taken by the patient exerted splanchnic vasoconstricting effects, which might reduce portal venous inflow or impair hepatic arterial buffer response^[1]. (4) Widespread atherosclerosis is commonly seen in diabetic and hypertensive patients, and it may cause the thickening of blood vessels and arteriostenosis. Abnormalities of hemodynamics, endothelial dysfunction and hypercoagulable state possibly leading to increased thrombosis and decreased fibrinolysis have been found in diabetic patients with angiopathy. These factors could contribute to the hepatic infarction in diabetes.

Hepatic infarction is usually accompanied by the outbreak of sudden upper abdominal pain, fever, elevated white blood cell count, and markedly elevated liver enzymes^[5]. Hyperglycemia may result in visceral autonomic nerve lesion and contribute to the insensitiveness to ischemic pain. This may explain why the patient did not feel abdominal pain.

Computerized tomography scan of upper abdomen in patients with hepatic infarction usually show a well-marginated peripheral zone without any contrast capture in the corresponding hepatic lobe, wedge-shaped and extending to the liver capsule^[4]. But in this case, liver computerized tomography scan exhibited untypical presentations, which led to the misdiagnosis of hepatic carcinoma and surgical operation and the diagnosis of hepatic infarction was finally established by liver pathology. MRI is the perfect method for the diagnosis of hepatic infarction because of its high resolution. In the early stage, the lesion shows slight long T₁ and slight long T₂ intensity. In the middle and late stages, it shows long T₁ and long T₂ intensity. The intensity is heterogenous due to various degrees of ischemia and infarction in the lesion. Generally speaking, the center of lesion is more apparent than the rim and shows prominent abnormal intensity as in the present case. The lesion shows no apparent enhancement and slight mass effect, which is the primary differentiation feature from hepatic mass.

In summary, we suggest that the diabetic patient with elevated liver enzymes should be suspected much of hepatic infarction and further liver computerized tomography scan should be considered to exclude the diagnosis.

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LETTERS TO THE EDITOR

Obstructive jaundice leads to accumulation of oxidized low density lipoprotein in human liver tissue

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Abstract

Oxidized low density lipoprotein (ox-LDL) molecule is one of the most important modified lipoproteins produced during the oxidative stress. Modified lipoproteins have been defined as being part of the immune inflammatory mechanisms in association with oxidant stress. We have reported the accumulation of ox-LDL in Balb/c mice liver after bile duct ligation previously. Here, we investigated this finding in human beings with obstructive jaundice. Our study demonstrates that obstructive jaundice results in tremendous accumulation of ox-LDL in the liver tissue of patients.

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Key words: Obstructive jaundice; Liver; Oxidative stress; Oxidized low density lipoprotein

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TO THE EDITOR

Oxidized low density lipoprotein (ox-LDL) molecule is one of the most important modified lipoproteins. Its popularity has arisen after our understanding about its

key role in the pathogenesis of atherosclerosis^[1]. These molecules have been defined as being part of the immune inflammatory mechanisms in association with oxidant stress^[2]. Hepatocyte injury during obstructive jaundice is involved in such pathways associated with oxidant stress. This association has been testified also by us in Balb/c mice. Moreover, our study demonstrated that tremendous ox-LDL is accumulated in the liver after bile duct ligation in mice^[3]. Furthermore, we have investigated this finding in human beings with obstructive jaundice.

The study was composed of two groups. One group enrolled 11 patients with various causes of obstructive jaundice, 5 percutaneous and 6 intraoperative wedge-resection liver biopsies were taken from these patients. The other group included 7 patients undergoing elective abdominal operations without primary hepatobiliary pathologies. Wedge-resection type liver biopsies were taken from all these patients. Routine liver biochemical parameters including cholestatic liver enzymes were studied. In liver biopsies stained with hematoxylin and eosin, we investigated cholestatic findings. Moreover, a special immunofluorescent staining was done for investigation of ox-LDL in the liver tissue by fluorescent microscopy. All patients in group 1 demonstrated various degrees of cholestasis. The patients in group 2 showed neither cholestasis nor any histopathologic abnormality in the liver biopsy samples. First anti-ox-LDL antibody and fluorescence - stained anti-anti ox-LDL antibody were applied on liver tissue samples as previously described^[3]. The patients in group 1 were found to have enormous accumulation of ox-LDL in the liver (Figure 1A). No patient in group 2 demonstrated ox-LDL deposition (Figure 1B).

In this investigation, we clearly demonstrated retention of ox-LDL molecules in the liver tissue of human beings with obstructive jaundice, which was also found in our mice study. As demonstrated in our mice study, enhanced lipid peroxidation in the liver due to oxidative stress in association with obstructive jaundice has been clearly notified by many authors^[4,5]. A major and early lipid peroxidation product is ox-LDL which is mainly filtered by liver macrophages and Kupffer cells^[6]. During the jaundice period after biliary obstruction, ox-LDL accumulates in the liver either because of its decreased secretion into the bile or because of enhanced production due to lipid peroxidation as the filtration capacity of Kupffer cells becomes insufficient. It is well known that ox-LDL molecules have specific receptors on hepatocytes, Kupffer

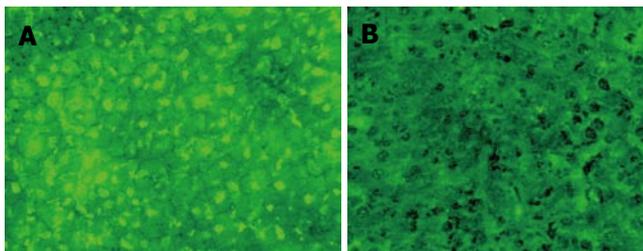


Figure 1 Enormous accumulation of oxidized low-density lipoprotein in patients of group 1 (A) and no oxidized low-density lipoprotein deposition in patients of group 2 (B) (x 100).

and stellate cells^[2,7]. The interaction of ox-LDL with these cells can lead to apoptosis of hepatocytes, enhancement of tumor necrosis factor- α and secretion of transforming growth factor- β by Kupffer cells, as well as transformation of stellate cells into collagen secreting myofibroblasts^[2,7,8]. Thus, it seems theoretically possible that the main lipid peroxidation byproduct, ox-LDL, may have important roles in liver consequences of biliary jaundice. Our study results indicate a concrete fact that ox-LDL accumulates in human liver tissue during obstructive jaundice. Nevertheless, further investigation should be done for more detailed analysis of such accumulation and its role in liver outcomes of obstructive jaundice.

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Meetings

MAJOR MEETINGS COMING UP

First Biennial Congress of the Asian-Pacific Hepato-Pancreato-Biliary Association March, 2007
Fukuoka, Japan
<http://www.congre.co.jp/1st-aphba>

American College of Gastroenterology Annual Scientific
20-25 October 2006
Las Vegas, NV

14th United European Gastroenterology Week, UEGW
21-25 October 2006
Berlin, Germany

APDW 2006: Asian Pacific Digestive Week 2006
26-29 November 2006
Lahug Cebu City, Philippines

EVENTS AND MEETINGS IN THE UPCOMING 6 MONTHS

Falk Symposium 151: Emerging Issues in Inflammatory Bowel Diseases
24-25 March 2006
Sydney - NSW
Falk Foundation e.V.
symposia@falkfoundation.de

10th International Congress of Obesity
3-8 September 2006
Sydney
Event Planners Australia
enquiries@ico2006.com
www.ico2006.com

Easl 2006 - the 41st annual
26-30 April 2006
Vienna, Austria
Kenes International

Prague hepatology 2006
14-16 September 2006
Prague
Foundation of the Czech Society of Hepatology
veronika.revicka@congressprague.cz
www.czech-hepatology.cz/phm2006

12th International Symposium on Viral Hepatitis and Liver Disease
1-5 July 2006
Paris
MCI France
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www.isvhld2006.com

Falk Symposium 152: Intestinal Disease Part I, Endoscopy 2006 - Update and Live Demonstration
4-5 May 2006
Berlin
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symposia@falkfoundation.de

Falk Symposium 153: Intestinal Disease Part II, Immunoregulation in Inflammatory Bowel Disease - Current Understanding and Innovation
6-7 May 2006
Berlin
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ILTS 12th Annual International Congress
3-6 May 2006
Milan
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22 July 2006-1 August 2006
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Hepatology
15-18 March 2006
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28 June 2006-1 July 2006
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International Conference on Surgical Infections, ICSI2006
6-8 September 2006
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www.icsi2006.se/9/23312.asp

7th World Congress of the International Hepato-Pancreato-Biliary Association
3-7 September 2006
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Society of American Gastrointestinal Endoscopic Surgeons
26-29 April 2006
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www.sages.org

Digestive Disease Week 2006
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Los Angeles
www.ddw.org

Annual Postgraduate Course
25-26 May 2006
Los Angeles, CA
American Society of Gastrointestinal Endoscopy
www.asge.org/education

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3-7 June 2006
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10th World Congress of the International Society for Diseases of the Esophagus
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Eradication of *H pylori* for the prevention of gastric cancer

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Abstract

Infection with *H pylori* is the most important known etiological factor associated with gastric cancer. While colonization of the gastric mucosa with *H pylori* results in active and chronic gastritis in virtually all individuals infected, the likelihood of developing gastric cancer depends on environmental, bacterial virulence and host specific factors. The majority of all gastric cancer cases are attributable to *H pylori* infection and therefore theoretically preventable. There is evidence from animal models that eradication of *H pylori* at an early time point can prevent gastric cancer development. However, randomized clinical trials exploring the prophylactic effect of *H pylori* eradication on the incidence of gastric cancer in humans remain sparse and have yielded conflicting results. Better markers for the identification of patients at risk for *H pylori* induced gastric malignancy are needed to allow the development of a more efficient public eradication strategy. Meanwhile, screening and treatment of *H pylori* in first-degree relatives of gastric cancer patients as well as certain high-risk populations might be beneficial.

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INTRODUCTION

Despite decreasing incidence and mortality rates, gastric cancer remains the second most frequent malignancy worldwide, with the majority of cases diagnosed at an advanced stage^[1]. A number of environmental factors, e.g.

diets high in salt and N-nitrosamines and low in fruits and vegetables have been shown to contribute to gastric cancer development^[2]. Furthermore, it is now well recognized that chronic infection with *H pylori* is tightly associated with the development of gastric cancer, primarily noncardiac gastric cancer. The clinical course of *H pylori* infection is highly variable and the likelihood of developing gastric cancer is determined by both microbial and host factors (Figure 1). Based on the large number of experimental and epidemiological studies, it seems reasonable to conclude that the eradication of *H pylori* should prevent gastric cancer. However, convincing results from clinical trials are not yet available. Hence, current clinical decision-making has to be based on indirect evidence: data from animal models and studies supporting the beneficial effect of eradication on the development of gastric cancer precursor lesions^[3]. This article reviews the existing evidence that *H pylori* eradication prevents gastric cancer with a highlight on recent publications relevant for the clinician.

PATHOGENESIS AND EPIDEMIOLOGY

Pathogenesis

According to Correa's model, gastric cancer development is a multistep process where the gastric mucosa is slowly transformed from normal epithelium to chronic gastritis, to multifocal atrophy, to intestinal metaplasia of various degrees, to dysplasia and finally to invasive cancer^[4]. However, this sequence of events does not precede diffuse type gastric cancer and has even been debated for the intestinal type^[5] since less than 10% of patients with these lesions ultimately develop gastric cancer^[6]. Most *H pylori* infected individuals show antral predominant gastritis, which predisposes them to duodenal ulcers, but rarely causes gastric cancer. On the contrary, patients with corpus-predominant gastritis are likely to develop gastric ulcers, gastric atrophy, intestinal metaplasia and eventually gastric cancer. Our group, among others, has found that the pattern and the morphological distribution of gastritis correlate strongly with the gastric cancer risk^[7,8]. We showed that the expression of *H pylori* associated gastritis in patients with gastric cancer is high in the corpus and is frequently associated with intestinal metaplasia and atrophy^[9]. Based on these findings we developed a gastric carcinoma risk index, which evaluates grade and activity of corpus-dominant *H pylori* gastritis as well as the occurrence of intestinal metaplasia in the antrum or corpus to determine a patient's risk for developing gastric carcinoma^[10]. In a subsequent case control study, the gastric carcinoma risk index had a sensitivity of 93% and a specificity of 85% for diagnosing individuals with gastric carcinoma^[11].

Epidemiology

Infection with *H pylori* occurs worldwide, but the prevalence varies greatly among countries and among different populations within the same country^[12]. The overall prevalence of *H pylori* infection is closely linked to current socioeconomic conditions^[13]. Although the incidence of the infection in industrialized countries has decreased substantially over recent decades, it will remain endemic for at least another century, unless intervention occurs^[14]. In the early 1990s a series of prospective case control studies^[15-18] demonstrated a close link between *H pylori* infection and gastric cancer, which prompted the World Health Organization to announce the bacterium a class I (definite) carcinogen in 1994. Since then data from various studies have accumulated that further strengthen the association between *H pylori* infection and gastric cancer. One of the most compelling studies was conducted in Japan, where Uemura *et al*^[19] prospectively followed 1526 patients over a period of 7.8 years. A total of 2.9 percent of *H pylori* infected individuals developed gastric cancer compared to none in the *H pylori* negative control group. Among individuals with *H pylori* infection, those with severe gastric atrophy (odds ratio: 4.9), corpus-predominant gastritis (odds ratio: 34.5) and intestinal metaplasia were at significantly higher risk for gastric cancer.

According to most retrospective, cohort and case control studies, the overall odds ratio for *H pylori* infection and gastric cancer is around two to six^[19-23]. However, these numbers are likely to represent a gross underestimation of the real risk. Among the confounding factors that make risk appear lower in most studies are the long latency between the initiation of the carcinogenic process and the clinical occurrence of cancer as well as the inclusion of individuals with antral predominant/duodenal ulcer phenotype^[24]. If selection of patients and methodology is optimized, the odds ratio for *H pylori* infected individuals may increase to a factor of around 20^[25-27].

RISK FACTORS

Bacterial virulence factors

H pylori displays a considerable amount of genetic variation. Even strains within an individual host commonly change over the course of the infection^[28,29]. A number of bacterial virulence factors have been discovered that influence disease outcome in infected individuals. The majority of *H pylori* strains express and secrete VacA, a vacuolating cytotoxin, which is inserted into the gastric epithelial-cell and mitochondrial membranes, possibly providing the bacterium with nutrients and inducing apoptosis of the host cell^[12,30]. VacA has also been found to modulate the host immune system *via* T-cell inhibition^[31,32]. Studies indicate that expression of VacA increases bacterial fitness and in some western countries VacA *s1* and VacA *m1* genotypes are associated with more severe forms of gastritis, atrophy, intestinal metaplasia and perhaps gastric cancer^[33-36]. Another major focus of research is the analysis of the *cag* pathogenicity island (*cag PAI*), a genomic fragment comprising 31 genes that support the translocation of the 120-kD CagA protein

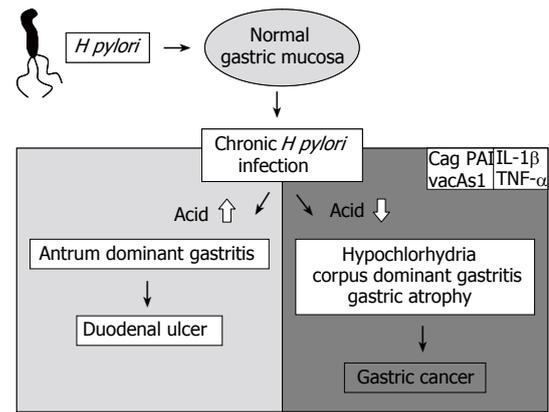


Figure 1 Variable course of *H pylori* infection.

into the gastric epithelial cell^[37,38]. CagA has been shown to induce cytokine production along with a growth factor-like response in the host cell and to disrupt the junction-mediated gastric epithelial cell barrier function^[39,40]. In western countries, patients carrying CagA+ *H pylori* strains are more likely to develop adenocarcinomas of the distal stomach than patients infected with CagA- strains^[41]. In particular, one recent meta-analysis of case-control studies concluded that infection with CagA+ strains increases the risk over *H pylori* infection alone^[42]. However, similar findings are not reported from Asia, where about 95% of all infected individuals carry CagA+ strains^[3,43,44].

Host genetic factors

H pylori leads to inflammation of the gastric mucosa in virtually all infected individuals. However, most *H pylori* infected humans do not develop gastric cancer even if they are infected with so-called more virulent strains, indicating that host factors play a crucial role. The fact that first-degree relatives of gastric cancer patients have a significantly increased risk for developing gastric cancer compared to patients without a family history further emphasizes the importance of genetic factors. For example, our group found some important gastric cancer related genes to be more prevalent in the gastric mucosa of first-degree relatives^[45-49].

The infection with *H pylori* triggers an extensive systemic and local inflammatory response. Gastric epithelial cells respond by producing enhanced levels of interleukin-1β, interleukin-2, interleukin-6, interleukin-8 and tumor-necrosis-factor-α^[50-52]. El-Omar and co-workers were the first to show that patients with certain Interleukin-1 gene cluster polymorphisms, which lead to enhanced production of the proinflammatory cytokine IL-1β are at increased risk for *H pylori* induced hypochlorhydria and gastric cancer^[53]. Further studies found that proinflammatory polymorphisms of the IL-1 receptor antagonist, tumor necrosis factor-α and IL-10 are also associated with an increased risk for the development of noncardia gastric adenocarcinoma^[54,55]. Interestingly, the combination of pro-inflammatory polymorphisms in the interleukin-1β gene and infection with more virulent *H pylori* strains seems to increase the gastric cancer risk even more^[56]. Most of the important studies exploring host

Table 1 Gastric cancer prevention studies

Study	Design	Follow-up	Patients	Treatment	Outcome
Uemura <i>et al</i> 1997	Nonrandomized intervention trial	2 yr	132 Japanese patients with endoscopically removed early stage gastric cancer and <i>H pylori</i> infection	<i>H pylori</i> eradication therapy or no treatment	Reduced rate of gastric cancer development after eradication of <i>H pylori</i>
Saito <i>et al</i> 2000	Nonrandomized intervention trial	2 yr	64 Japanese patients with gastric adenoma and <i>H pylori</i> infection	<i>H pylori</i> eradication therapy or no treatment	Reduced rate of metachronous gastric cancer development after eradication of <i>H pylori</i>
Correa <i>et al</i> 2000	Prospective, randomized, placebo controlled trial	6 yr	852 individuals from a high risk region in Colombia with <i>H pylori</i> infection and precancerous lesions	<i>H pylori</i> eradication therapy and/or ascorbic acid/beta-carotene or placebo	Significant increase in the rate of regression of precursor conditions after cure of <i>H pylori</i> and/or treatment with dietary supplements
Wong <i>et al</i> 2004	Prospective, randomized, placebo controlled trial	8 yr	1630 individuals from a high risk region in China with <i>H pylori</i> infection; with or without precancerous lesions	<i>H pylori</i> eradication therapy or placebo	Significant reduction in gastric cancer risk after cure of <i>H pylori</i> only for patients without precancerous conditions
Take <i>et al</i> 2005	Nonrandomized intervention trial	3.4 yr	1342 Japanese patients with peptic ulcer disease	<i>H pylori</i> eradication therapy	Significant increase in gastric cancer risk for patients with persistent <i>H pylori</i> infection

genetics were performed in Caucasian populations and still need to be confirmed in other ethnic groups^[3]. However, there is emerging evidence that similar associations can be found in Asian populations. For example, one study from Japan showed that proinflammatory IL-1 β polymorphisms in *H pylori* infected Japanese individuals are significantly associated with hypochlorhydria and atrophic gastritis^[57]. Recent data by Goto *et al*^[58] also indicate that a common polymorphism in the coding gene for SHP-2 that interacts with the CagA protein can increase the risk for gastric atrophy in Japanese patients infected with CagA+ *H pylori* strains. The authors speculate that this might explain why only a proportion of CagA+ individuals develop gastric atrophy even though this strain is almost universal in Asian countries.

IN VIVO STUDIES

Animal models

A number of animal models have been developed to study the mechanisms by which *H pylori* induces gastric carcinogenesis. Using the Mongolian gerbil model, several studies provided evidence that *H pylori* infection is in fact a potent carcinogen and able to induce gastric cancer by itself^[59-62]. The studies by Watanabe *et al*^[59] and Honda *et al*^[60] found that 37% and 40% of infected animals developed well-differentiated intestinal adenocarcinomas 62 and 72 wk after inoculation of the bacterium. Both studies used *cagA* and *vacA* positive *H pylori* strains for infection of the animals. The risk of gastric carcinogenesis in Mongolian gerbils increases significantly through combination of *H pylori* infection with other known carcinogens such as N-methyl-N-nitrosourea (NMU) and N-methyl-N-nitro-N-nitrosoguanidine (MNGG)^[63-65]. Studies using *H pylori* or *H. felis* infected mice found that the gastric cancer development is strongly determined by host specific factors, for example specific patterns of immune response. Some mouse strains develop a vigorous

Th-1 response to the infection while others have a predominant Th-2 immune response and seem to be more resistant to mucosal damage. Those with the strong TH-1 response continue to develop atrophy, metaplasia and eventually invasive cancer in a gender specific manner^[66].

There is evidence from animal models, that eradication of *H pylori* is able to prevent gastric carcinogenesis. The incidence of gastric adenocarcinoma in nitrosamine administered Mongolian gerbils with *H pylori* infection was significantly lower in animals receiving *H pylori* eradication^[67,68]. Mouse models have also provided important evidence of beneficial effects from the administration of anti-inflammatory drugs, where atrophy and metaplasia have been reversed, in some cases completely^[69].

Clinical studies

Cure of *H pylori* infection results in several physiologic effects that are likely to reduce gastric cancer risk. These include reduction in cell turnover, elimination of DNA damage by a reduction of reactive oxygen species, increased gastric acid secretory capacity and restoration of ascorbic acid secretion into the gastric juice^[1,70,71]. However, evidence from well-designed clinical studies supporting the cancer protective effect of *H pylori* eradication remains sparse (Table 1). Among the first clinical data to support the hypothesis that *H pylori* eradication is able to prevent gastric cancer development were case-control studies from Sweden on patients undergoing hip replacement procedures. Akre *et al*^[72] showed that significantly reduced rates of gastric cancer occurred in such patients who frequently receive high doses of prophylactic antibiotics, incidentally eradicating *H pylori* infection. As discussed earlier in this review, the study by Uemura *et al*^[19] provides some of the strongest evidence for the causative role of *H pylori* infection in gastric cancer development. Here, gastric cancer developed in 2.9% of all *H pylori* infected patients compared with 0% of those without infection. Notably,

no case of cancer developed in a subgroup of 253 *H pylori* infected patients who received eradication therapy at an early time point after enrollment in the study. The same group of investigators found that eradication of *H pylori* was able to prevent relapse after endoscopic resection of early stage gastric cancer^[73]. Another study by Saito *et al*^[74] showed that *H pylori* eradication had a favorable impact on gastric cancer development in patients with gastric adenoma. More recently, Take *et al*^[75] published the results from a large prospective Japanese intervention trial. The authors endoscopically followed 1342 patients with peptic ulcer disease for a mean period of 3.4 years. All patients initially received *H pylori* eradication therapy. The risk of developing gastric cancer was significantly higher in the group of patients who failed eradication therapy compared to those who were cured for the infection.

The first prospective randomized controlled study to examine the effect of *H pylori* eradication on gastric cancer development was published by Wong *et al*^[76] in 2004. The authors randomized 1630 individuals from a high-risk region in China with confirmed *H pylori* infection to eradication therapy or placebo. After a follow-up period of 7.5 years, they found no difference in gastric cancer incidence between those receiving *H pylori* eradication therapy and those who were not given treatment (7 *vs* 11 cases, *P* = 0.33). However, further subgroup analysis of the data demonstrated a significant benefit (*P* = 0.02) from eradication therapy in patients without baseline intestinal metaplasia at the time of study enrollment.

Unfortunately, several international prospective randomized controlled trials, designed to evaluate the long-term effect of *H pylori* eradication on gastric cancer development had to be abandoned. For example, the PRISMA study^[77], initiated in 1998 by our group to test the effect of *H pylori* eradication therapy in a high-risk population in Germany and Austria was discontinued due to insufficient recruiting. As might be expected, most eligible patients for those studies are not willing to enter the placebo arm after the nature of such a trial has been explained to them. Apart from ethical issues, the required follow-up time of 10 to 20 years for these trials remains an additional problem. A growing number of studies are therefore using surrogate markers for gastric cancer development, namely gastric atrophy and intestinal metaplasia as primary study endpoints.

There is consistent evidence that *H pylori* eradication cures gastritis and numerous studies have shown that atrophy and metaplasia do not progress in patients after *H pylori* eradication compared to control groups who remain *H pylori* positive^[78-86]. However, many of the available studies addressing the reversibility of gastric atrophy and intestinal metaplasia have yielded conflicting and inconsistent results, possibly because most of them are nonrandomized, not controlled, have short follow-up periods and only include small numbers of patients^[1,3]. One of the few randomized controlled trials for the prevention of gastric dysplasia was conducted by Correa *et al*^[87] in 2000. The authors found significant regression of gastric atrophy and intestinal metaplasia after *H pylori* eradication alone and in combination with β -carotene and ascorbic acid. Sung *et al*^[88] prospectively followed a total

of 587 *H pylori* infected patients, randomized to receive either eradication therapy or placebo, endoscopically for one year. Decrease in acute and chronic gastritis was significantly more frequent after *H pylori* eradication, but after the relatively short follow-up period, changes in intestinal metaplasia were similar between the two groups. The majority of available studies suggest, however, that regression of atrophic gastritis and, to a lesser extent, intestinal metaplasia can occur at least in a subset of patients with sufficient follow-up^[3,89].

In conclusion, there is little randomized controlled trial evidence to suggest that *H pylori* eradication decreases the risk of gastric cancer development. However, regression of gastric cancer precursor lesions may occur in some patients. At present, there are no markers that help to predict such a response in the individual patient. Therefore, eradication at the earliest possible time point in the disease process seems favorable. The optimal age for testing of *H pylori* infection still needs to be determined but available data suggest that eradication at a younger age might be a more favorable approach. Future research has to focus on identification of host and bacterial specific markers that will help to predict the development of gastric cancer in the *H pylori* infected individuals. Better identification of individuals at high risk for gastric cancer will allow for more effective prevention and eradication strategies. Meanwhile, screening and treatment of *H pylori* in first-degree relatives of gastric cancer patients as well as certain high-risk populations might be beneficial.

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EDITORIAL

Endoscopic submucosal dissection for stomach neoplasms

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Abstract

Recent advances in techniques of therapeutic endoscopy for stomach neoplasms are rapidly achieved. One of the major topics in this field is endoscopic submucosal dissection (ESD). ESD is a new endoscopic technique using cutting devices to remove the tumor by the following three steps: injecting fluid into the submucosa to elevate the tumor from the muscle layer, pre-cutting the surrounding mucosa of the tumor, and dissecting the connective tissue of the submucosa beneath the tumor. So the tumors are resectable in an *en bloc* fashion, regardless of the size, shape, coexisting ulcer, and location. Indication for ESD is strictly confined by two aspects: the possibility of nodal metastases and technical difficulty, which depends on the operators. Although long-term outcome data are still lacking, short-term outcomes of ESD are extremely favourable and laparotomy with gastrectomy is replaced with ESD in some parts of therapeutic strategy for early gastric cancer.

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Key words: Therapeutic endoscopy; Endoscopic submucosal dissection; Stomach neoplasia; Early cancer; Node-negative tumor

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INTRODUCTION

Endoscopic resection of stomach neoplasms has been originated from the development of a polypectomy technique using the high-frequency current to gastric polyps in 1968^[1,2], and it has become popular as endoscopic mucosal

resection (EMR) since the birth of a strip biopsy method in 1984^[3] and a cap technique in 1993^[4]. Endoscopic submucosal dissection (ESD) technique is a new endoscopic treatment using cutting devices, which has developed from one of the EMR techniques, namely endoscopic resection after local injection of a solution of hypertonic saline-epinephrine (ERHSE)^[5]. ESD consists of the following three steps: injecting fluid into the submucosa to elevate the tumor, pre-cutting the surrounding mucosa of the tumor, and dissecting the connective tissue of the submucosa beneath the tumor^[6-10]. Major advantages of this technique in comparison with conventional EMR are as follows. The resected size and shape can be controlled, *en bloc* resection is possible even in a large tumor, and tumors with ulcerative findings are also resectable. So this technique can be applied to the resection of complex tumors such as large tumors, ulcerative non-lifting tumors, and recurrent tumors. The disadvantages of this technique are requirement of 2 or more assistants, time-consuming, much more bleeding and a little higher perforation rate than conventional EMR^[11]. It is still controversial whether the esophageal or colorectal neoplasms in an early stage should be resected in an *en bloc* fashion by using ESD, considering the technical difficulty, associated risks, and favorable outcomes by conventional EMR^[12-16]. However, in case of the stomach neoplasms, especially when large or ulcerative tumors are targeted as the subjects of endoscopic resection, necessity of *en bloc* resection is emphasized, because multi-fragmental resection causes insufficient histological evaluation and local recurrence of multi-fragmental resection becomes significantly higher than that of *en bloc* resection^[17]. So in Japan, ESD is now gaining acceptance as the standard endoscopic resection techniques for stomach neoplasms in an early stage, especially for large or ulcerative tumors.

INDICATION FOR ENDOSCOPIC SUBMUCOSAL DISSECTION

Although institutional differences in indications for endoscopic resection have existed for a long time, empirical indication for conventional EMR is differentiated-type of mucosal cancers without ulcerative findings, with ≤ 2 cm in size if elevated or ≤ 1 cm in size if depressed or flat^[18]. The Japanese Gastric Cancer Association issued their gastric cancer treatment guidelines in 2001, showing that differentiated-type of mucosal cancers without ulcerative findings, with ≤ 2 cm in size, regardless of the tumor morphology are practically indicated for endoscopic resection^[19]. These criteria are determined by considering two aspects: the conditions free of lymph node metastasis and

Table 1 Frequency of lymph node metastases in early gastric cancer^[20]

Criteria	Frequency (No. with metastasis/total number)	95% CI
Intramucosal cancer differentiated adenocarcinoma, no lymphatic vascular invasion, irrespective of ulcer findings, tumor ≤ 3 cm	0/1230	0-0.3%
Intramucosal cancer differentiated adenocarcinoma, no lymphatic vascular invasion, without ulcer findings, irrespective of tumor size	0/929	0-0.4%
Intramucosal cancer undifferentiated adenocarcinoma, no lymphatic vascular invasion, without ulcer findings, tumor ≤ 2 cm	0/141	0-2.6%
Cancer with minute submucosal penetration (≤ 500 μm) differentiated adenocarcinoma, no lymphatic vascular invasion, irrespective of ulcer findings, tumor ≤ 3 cm	0/145	0-2.5%

the probability of successful *en bloc* resection. If the technical problems are overcome, indication could be expanded for all tumors which have been elucidated as node-negative tumors in clinical trials (Table 1)^[20].

TECHNIQUES OF ENDOSCOPIC SUBMUCOSAL DISSECTION

ESD requires special cutting knives, such as a needle knife^[5], an insulation-tipped electro-surgical (IT) knife^[6,21-24], a hook knife^[25,26], a flex knife^[8-10], and a triangle-tip (TT) knife^[27,28], or special devices such as a small-caliber tip transparent (ST) hood^[29] (Figure 1). As another approach to successful ESD, investigations of submucosal injection solutions have been actively done. It was reported that a hyaluronic acid solution makes a better long-lasting submucosal cushion without tissue damage than other available solutions^[29-33]. As a further improvement of hyaluronic acid solution, usefulness of a mixture of high-molecular-weight hyaluronic acid, glycerin, and sugar has also been reported^[34,35]. A representative case of ESD procedure using a flex-knife and a mixture of high-molecular-weight hyaluronic acid, glycerin, and sugar is shown in Figure 2^[36].

Marking around the tumor

After chromoendoscopy using 0.25% indigo carmine to clarify the border of the tumor, circumferential markings are made by using a flex-knife, which is set to about 1 mm in length of the knife, at about 5 mm outside of the tumor with 2-mm intervals between each marking.

Submucosal injection

Solutions prepared for submucosal injection are a 10% glycerine with 0.9% NaCl plus 5% fructose solution (glyceol, Chugai Pharmaceutical co., Tokyo Japan) alone for small distal gastric tumors without ulcer findings and a mixture of glyceol and 1% 1900 ku hyaluronic acid preparation (suvenyl, Chugai Pharmaceutical co. Tokyo Japan) for complex or proximal gastric tumors. The mixing ratio of glyceol and suvenyl is 7:1 for complex or proximal gastric tumors. A small amount of epinephrine to make a concentration of 0.0005% is added to obtain vasoconstriction for hemostasis and indigo carmine is also added to find out the seeping area of the submucosal injection solution as operators' preference. Injection of these solutions is performed into the submucosal layer just outside the markings where mucosal incision intends to be made

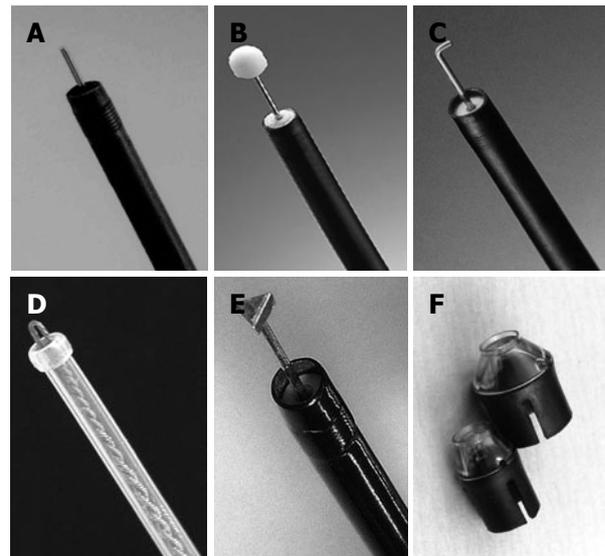


Figure 1 Devices for ESD. **A:** Needle knife (KD-1L-1, Olympus, Tokyo, Japan); **B:** IT (KD-610L, Olympus, Tokyo, Japan); **C:** Hook knife (KD-620LR, Olympus, Tokyo, Japan); **D:** Flex knife (KD-630L, Olympus, Tokyo, Japan); **E:** TT knife (KD-640L, Olympus, Tokyo, Japan); **F:** ST hood (DH-15GR, 15CR, Fujinon Toshiba ES Systems, Tokyo, Japan).

at first. The volume of injection is about 2 mL once, and injection is repeated several times before starting mucosal incision until the targeted area is lifted enough. After exposure of the submucosal layer, injection is applicable from the exposed submucosal layer to lift up the submucosal layer intended to be cut.

Mucosal incision

After the tumor is lifted, mucosa outside the markings is incised circumferentially by using the same flex-knife, which is set to about 2 mm in length of the knife. The starting point for cutting is principally a distal part from the mouth. Retroflex position of the endoscope is usually used if applicable when the distal part of the tumor is cut.

Submucosal dissection

Small tumors can be resected by an electro-surgical snare only after mucosal incision around the markings without submucosal dissection. However, large tumors and tumors with ulcer findings or located in a tortuous area cannot be resected by an electro-surgical snare, dissecting the submucosa completely is thus needed. A flex-knife, which is set to about only 1 or 2 mm in length of the knife is also

Table 2 Outcomes of ESD

Techniques	En bloc resection rate %		Local recurrent rate %	Complication rate %	
	≤ 20 mm	> 21 mm		Bleeding	Perforation
ESD with IT knife ^[42]	95 (686/719)	86 (271/314)	NA	6 (59/945)	4 (35/945)
ESD with the tip of an electro-surgical snare (thin type)/a flex knife ^[8]	95 (56/59)		NA	1.7 (1/59)	3.4 (2/59)
ESD with sodium hyaluronate and small-caliber-tip transparent hood ^[43]	100 (37/37)	97 (32/33)	NA	1 (1/70)	0 (0/70)
ESD with a hook knife ^[44]	95 (194/204)		0.5 (1/204)	NA	1.5 (3/204)
Submucosal-endoscopic resection with hypertonic saline-epinephrine solution (S-ERHSE) ^[45]	NA	79 (36/46)	0 (0/46)	4(2/46)	8 (4/46)
ESD with a mixture of high-molecular-weight hyaluronic acid and Glyceol ^[35]	100 (26/26)		0 (0/26)	3.8 (1/26)	0 (0/26)
ESD with TT knife ^[28]	88 (14/16)		NA	NA	0 (0/16)

NA: Not analyzed.

used for dissecting the submucosa. If the target to be dissected cannot be seen directly by any way, a transparent attachment on the tip of the endoscope is useful to stretch the connective tissue and improve the field of viewing in the submucosa. Because the submucosal cushion flattens down as time is passed, it is also important to start dissecting the submucosa immediately from the incising part of the mucosa before further marginal mucosal cutting and to inject the prepared solution into the submucosa repeatedly if a security cushion is necessary to be kept.

Effective control of bleeding during the procedure, especially in the step of submucosal dissection is important for safer, faster, and more reliable ESD. It is more desirable to prevent bleeding than to stop it after its occurrence. For vessels, which are smaller than the tip of the knife, non-contact electrocoagulation of the vessels with a flex-knife is usually enough to prevent or stop bleeding without changing to another device. Bleeding from large vessels also can be prevented or stopped by using a hemostatic forceps.

Management of post ESD ulcer

After total removal of the tumor, all visible vessels located in the post-ESD ulcer base are treated using hemostatic forceps. Finally, 20 mL of sucralfate liquid is sprayed using the outer sheath of a rotational endoscopic clip device to confirm the achievement of correct hemostasis and to coat the post-procedure ulcer base^[37].

Management after ESD

After ESD, patients are prohibited from eating and drinking until the next day of ESD. If laboratory findings and chest and abdominal X-ray remain unremarkable, the patients are permitted oral soft foods. Follow-up endoscopy is performed within 1 wk to check up post ESD ulcer healing before the patient is discharged from the ward. Proton pump inhibitor and sucralfate are administered until confirmation of healing of the post ESD ulcers. All patients with ESD also undergo a follow-up endoscopy 2 mo after ESD to confirm the healing and exclude recurrence^[38-40]. In case of curative ESD *en-bloc* resection, annual endoscopies are performed to detect new metachronous tumors rather than recurrent tumors, since the local recurrence rate is very low^[17]. For tumors with non-curative or

non-evaluable resection margins, but fulfilling the criteria of node-negative tumors, endoscopy is performed every 6 mo to detect local recurrent tumors, at least for the first three years of follow-up.

Outcomes of endoscopic submucosal dissection

In comparison with outcomes of conventional EMR (approximately 75% of the *en bloc* resection rate, a high risk of local recurrence ranging from 2% to 35%^[41], those of ESD are extremely good. As shown in Table 2, the *en bloc* resection rate is more than 90% regardless of the size while the local recurrence rate is almost zero^[8,28,35,42-45].

Complications of endoscopic resection include pain, bleeding, perforation, stricture formation, *etc.* Bleeding is the most common complication, which is typically minor and treatable with endoscopy. The risks vary according to the definition of bleeding (Table 2). Most bleeding occurs during the procedure or within 24 h and predominantly in tumors located in the upper third during the procedure or the lower third of the stomach after the procedure^[42]. Our preliminary data show that 13 of 382 resections (3.4%) are complicated by post-ESD bleeding. Eight bleedings (62%) occur from the post-ESD ulcers located in the lower third, 4 from the middle third and 1 from the upper third of the stomach. Eight bleedings (62%) also occur within 24 h after ESD, 2 d after ESD, 3 between 6 d and 10 d after ESD. These findings indicate that post-ESD bleeding should be especially checked for within a day after ESD when the tumors are located in the lower third of the stomach.

Perforation is another major complication related to ESD. As a result of the techniques, the perforation rate of ESD has decreased to 0%-8%^[7,22,25,28,35,42,43] (Table 2). Furthermore, recent case series suggest that immediately recognized perforation can be successfully sealed with endoclips as conservatively observed without emergency laparotomy by endoscopic clipping, nasogastric suction, decompression of pneumoperitoneum, and antibiotics^[46].

CONCLUSION

Endoscopic resection has become a reasonable and convenient diagnostic and treatment modality, because

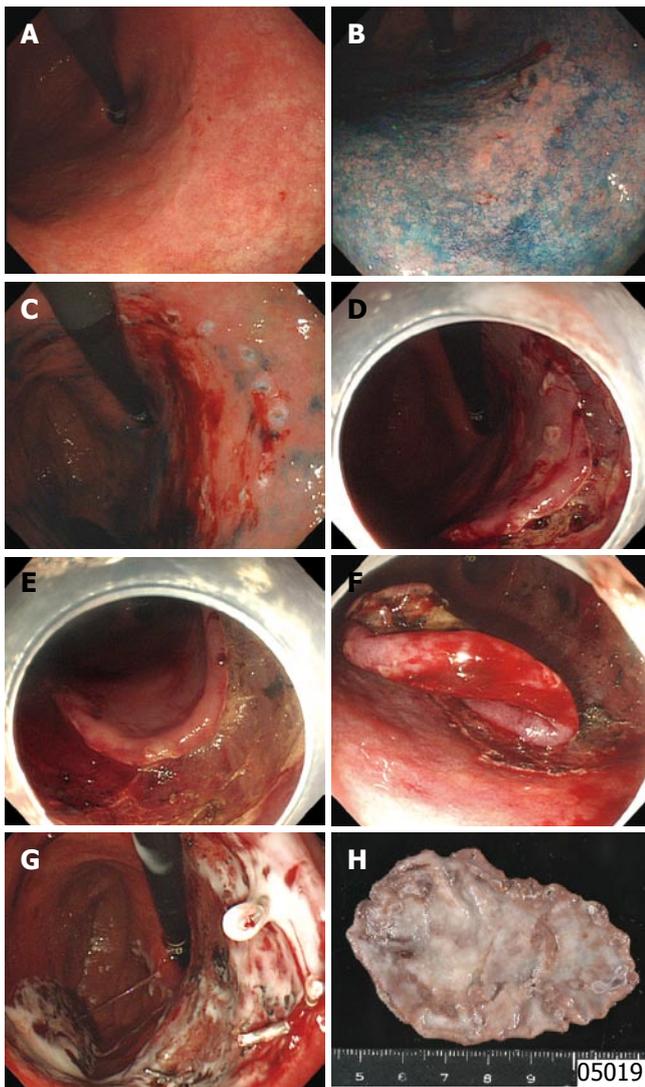


Figure 2 Endoscopic submucosal dissection (ESD). **A:** Ordinal endoscopy showing a whitish slight elevation with a blurred margin in the lesser curvature of the middle gastric body; **B:** Chromoendoscopy revealing margins of the lesion clearly; **C:** Marking dots on the circumference of the lesion; **D:** The incised mucosa around the marking dots of the distal margins; **E:** Before completion of circumferential mucosal incision, submucosal dissection from the distal edges; **F:** After mucosal incision with slight submucosal dissection circumferentially, submucosal dissection from the edge of the posterior wall to the anterior wall; **G:** Complete detachment of the lesion from the muscle layer and spraying sucralfate for confirmation of hemostasis; **H:** The resected specimen including the whole marking dots showing *en bloc* resection of the lesion.

histological information about the whole tumor can be obtained and furthermore, a curative treatment is achieved in case of localized tumors without lymph node metastasis, preserving the whole stomach. From this point of view, ESD enables us to resect not only small tumors, but also large or ulcerative tumors endoscopically. ESD has also brought us the concept of diagnostic endoscopic resection for some tumors clinically diagnosed as submucosal invasive cancers, because histopathological diagnosis of submucosal invasive cancers lacks consistency with clinical diagnosis in 66% of cases^[47]. If we can perform thorough and precise histopathological investigations using the resected specimens in an *en bloc* fashion, there is no way to deny the application of

endoscopic resection as the first step before gastrectomy, which would consequently avoid unnecessary gastrectomy. However, some complications related to ESD are still a matter of concern. Further refinements of the technique will ultimately help to achieve the goal of eradication of stomach neoplasms.

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Cyclooxygenases in hepatocellular carcinoma

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Abstract

Many epidemiological studies demonstrate that treatment with non-steroidal anti-inflammatory drugs (NSAIDs) reduce the incidence and mortality of certain malignancies, especially gastrointestinal cancer. The cyclooxygenase (COX) enzymes are well-known targets of NSAIDs. However, conventional NSAIDs non-selectively inhibit both the constitutive form COX-1, and the inducible form COX-2. Recent evidence indicates that COX-2 is an important molecular target for anticancer therapies. Its expression is undetectable in most normal tissues, and is highly induced by pro-inflammatory cytokines, mitogens, tumor promoters and growth factors. It is now well-established that COX-2 is chronically overexpressed in many premalignant, malignant, and metastatic cancers, including hepatocellular carcinoma (HCC). Overexpression of COX-2 in patients with HCC is generally higher in well-differentiated HCCs compared with less-differentiated HCCs or histologically normal liver, suggesting that COX-2 may be involved in the early stages of hepatocarcinogenesis, and increased expression of COX-2 in noncancerous liver tissue has been significantly associated with shorter disease-free survival in patients with HCC.

In tumors, overexpression of COX-2 leads to an increase in prostaglandin (PG) levels, which affect many mechanisms involved in carcinogenesis, such as angiogenesis, inhibition of apoptosis, stimulation of cell growth as well as the invasiveness and metastatic potential of tumor cells.

The availability of novel agents that selectively inhibit COX-2 (COXIB), has contributed to shedding light on the role of this molecule. Experimental studies on animal models of liver cancer have shown that NSAIDs, including both selective and non-selective COX-2 inhibitors, exert

chemopreventive as well as therapeutic effects. However, the key mechanism by which COX-2 inhibitors affect HCC cell growth is as yet not fully understood.

Increasing evidence suggests the involvement of molecular targets other than COX-2 in the anti-proliferative effects of COX-2 selective inhibitors. Therefore, COX-inhibitors may use both COX-2-dependent and COX-2-independent mechanisms to mediate their antitumor properties, although their relative contributions toward the *in vivo* effects remain less clear.

Here we review the features of COX enzymes, the role of the expression of COX isoforms in hepatocarcinogenesis and the mechanisms by which they may contribute to HCC growth, the pharmacological properties of COX-2 selective inhibitors, the antitumor effects of COX inhibitors, and the rationale and feasibility of COX-2 inhibitors for the treatment of HCC.

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Key words: Cyclooxygenase-2; Cyclooxygenase-1; Hepatocellular carcinoma; Non-steroidal anti-inflammatory drugs; Inhibit cyclooxygenase-2

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INTRODUCTION

Hepatocellular carcinoma is one of the most common malignancies worldwide, accounting for approximately 6% of all human cancers and 1 million deaths annually, with an estimated number of new cases of over 500 000 per year^[1,2]. Although the clinical diagnosis and management of early-stage hepatocellular carcinoma (HCC) has improved significantly, HCC prognosis is still extremely poor and the cellular mechanisms contributing to hepatic carcinogenesis are relatively unknown. Therefore, investigating HCC pathogenesis and finding new diagnostic and treatment strategies is important.

Various risk factors have been associated with HCC, such as hepatitis B (HBV) and hepatitis C (HCV) viral infections, alcohol consumption and aflatoxin B1 (AFB1) intake. HBV and HCV infections are the most frequent underlying causes of HCC. However, although a number of experimental observations underline the

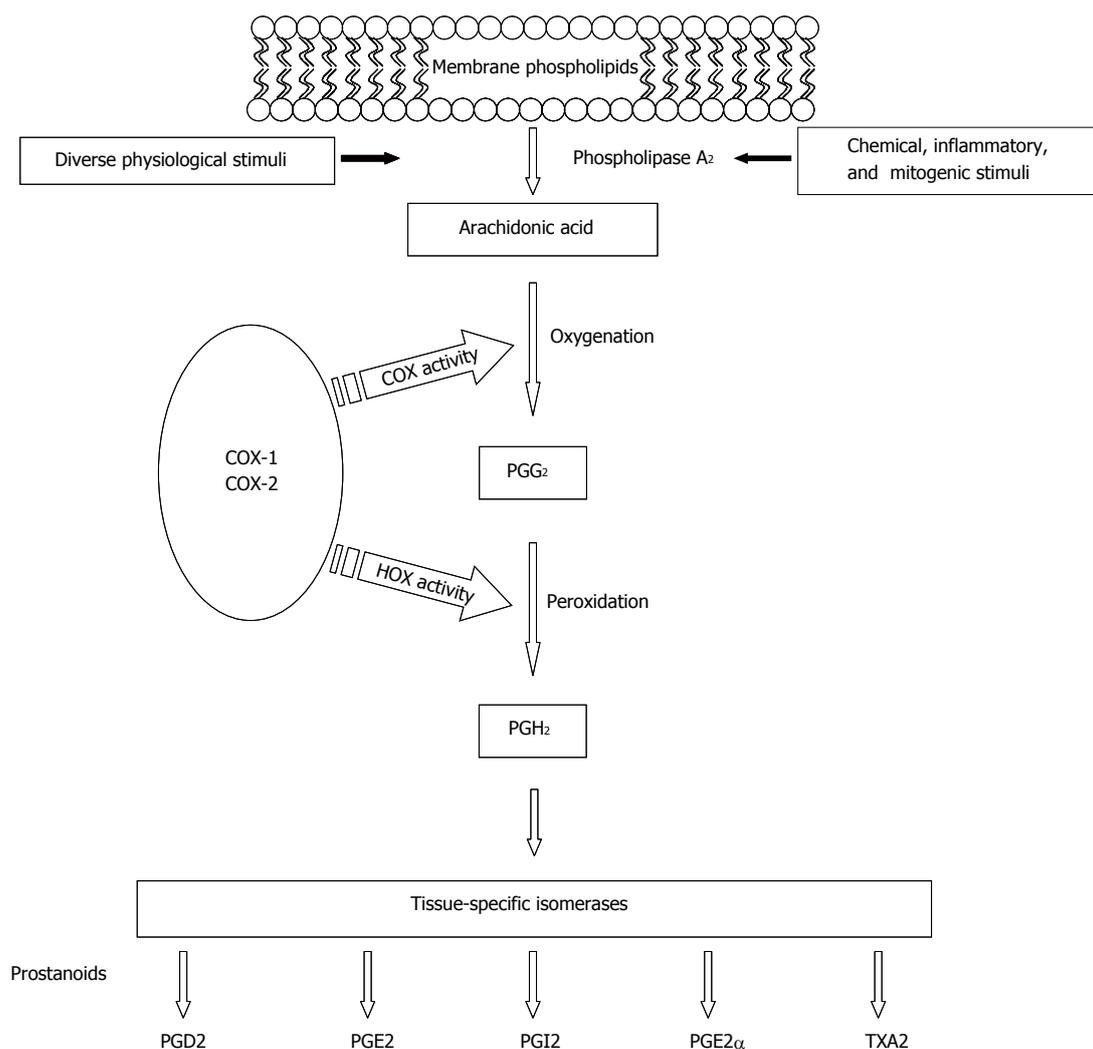


Figure 1 Prostanoids biosynthetic pathway.

potential for viral products in contributing to hepatocyte transformation, only in a minority of patients among the many suffering from chronic viral hepatitis and cirrhosis is there a neoplastic transformation in a given time lapse, suggesting that other co-oncogenic events are probably involved in the multistep process of hepatocyte transformation *in vivo*. HCC development is in fact a complex process associated with an accumulation of genetic and epigenetic changes that pass through the steps of initiation, promotion and progression.

Chronic inflammation is a recognized risk factor for carcinogenesis. Indeed it is thought to play a role in the pathogenesis of several types of cancers, such as cervical cancer, ovarian cancer, oesophageal adenocarcinoma, mesothelioma, colorectal cancer, lung cancer and also HCC^[3]. The ability of inflammation alone to cause malignancy is supported by the fact that other non-viral, inflammatory diseases of the liver such as alcoholic hepatitis, hemochromatosis, and primary biliary cirrhosis can also predispose to the development of hepatocellular carcinoma. Therefore, hepatic inflammation, due to viral and also non-viral chronic liver diseases, may represent an early step in the development of malignancy with genetic changes occurring as a later manifestation of a prolonged

(chronic) inflammatory process. Inflammatory-mediated events, such as the production of cytokines, reactive oxygen species (ROS), and mediators of the inflammatory pathway, such as cyclooxygenase-2 (COX-2), may therefore contribute to tumor formation. Recent evidence indicates that COX-2 is an important molecular target for anticancer therapies, and COX-2 inhibitors appear to have anticancer effects in different types of malignancies.

FUNCTIONS AND STRUCTURE OF THE CYCLOOXYGENASES

At least two distinct cyclooxygenases are present in humans, COX-1 and COX-2. COX enzymes, also referred to as prostaglandin H synthases, or prostaglandin endoperoxide synthases, are the rate-limiting enzymes that catalyze prostaglandin (PG) and thromboxane (TX) synthesis from 20 carbon polyunsaturated fatty acids, most commonly arachidonic acid (AA), which are released from membrane-bound phospholipids, usually by the action of phospholipase enzyme A₂ (Figure 1). Next, oxygenation of AA by COX produces an unstable intermediate, prostaglandin G₂ (PG₂), which is converted to prostaglandin H₂ (PGH₂) by the peroxidase activity

of COX. PGH₂ is subsequently converted to other PGs (PGD₂, PGE₂, PGF₂α, PGI₂) or thromboxanes (TXA₂). The array of PGs produced varies according to the downstream enzymatic machinery present in a particular cell type (Figure 1).

COX enzymes are proteins with a molecular weight of about 68 kilodaltons (kDa) in an unmodified condition, which increases to 72-74 kDa after post-translation glycosylation^[4]. The structure of COX enzymes consists of three distinct domains: an N-terminal domain with a conformation that is highly similar to that of epidermal growth factor, a domain containing a series of amphipathic helices, which comprise the membrane attachment site, and a C-terminal catalytic domain, which contains the cyclooxygenase and peroxidase active sites.

Although the two enzymes are highly similar in structure and enzymatic activity they have different genomic structures and different gene regulations and expressions. COX-1 was first purified and characterized in the 1970s and the gene was isolated in 1988^[5-7], whereas the COX-2 gene was cloned in 1993^[8]. COX-1 and COX-2 are encoded by separate genes located on different human chromosomes. The gene encoding for COX-1 enzyme is located on chromosome 9 (9q32-9q33.3) and is approximately 40 kilobase (kb) pairs, contains 11 exons and its mRNA is 2.8 kb^[9]. The gene encoding for COX-2 is located on chromosome 1 (1q25.2-25.3), contains 10 exons and is approximately 8.3 kb with a 4.5 kb transcript^[10].

The COX-1 gene exhibits the features of a housekeeping gene, it lacks a TATA box^[11], and is generally not subject to transcriptional induction, but it is constitutively expressed with near-constant levels and activity in most tissues and cell types.

COX-2 is an inducible or early-response gene, whose expression is undetectable in most normal tissues. COX-2 is highly induced in response to a broad spectrum of stimuli such as bacterial lipopolysaccharide (LPS)^[12], cytokines^[13], and growth factors^[14,15]. The inducibility of COX-2 can be explained by the presence, in the 5' -flanking region of its gene promoter, of several potential transcription regulatory sequences, including a TATA box and multiple transcription factor binding sites (C/EBP, AP-2, SP1, NF-κB, CRE, Ets-1, PEA-3 and GATA-1)^[16,17]. Transcriptional control of the COX-2 gene is cell-specific, and it is evident that more than one pathway may cooperate to regulate COX-2 expression. As reported by Araki^[18], in human hepatocellular carcinoma cells, increased COX-2 mRNA and protein expression may result from the combined de-regulation of Wnt and Ras pathways. In addition, in the adult liver, hepatocytes show a behavior pattern unique among cells that respond to inflammatory stresses. In contrast to fetal hepatocytes, which express COX-2 in response to proinflammatory stimuli^[19], such as LPS and proinflammatory cytokines, adult hepatocytes fail to express COX-2 regardless of the type of challenge^[20]. The presence of high levels of C/EBP-α seems to be involved in the impairment of COX-2 expression in these cells when challenged with proinflammatory stimuli^[20]. Therefore, the expression of COX-2 associated with liver diseases, such as cirrhosis and HCC, could be considered a marker of dedifferentiation in adult hepatocytes.

COX-2 gene expression is also subject to negative regulation. Indeed, COX-2 expression can be inhibited by glucocorticoids, IL-4, IL-13 and the anti-inflammatory cytokine IL-10^[21-23].

COX-2 expression can also be regulated at post-transcriptional levels in tumors. In the 3' untranslated region (3'-UTR) of the COX-2 mRNA there are multiple copies of the AUUUA motif, which are known to be involved in the control of both mRNA stability and protein translation. Such motifs represent potential targets by which various agents can stabilize or destabilize the COX-2 mRNA, and this may ultimately lead to an increase or decrease in enzyme activity levels. It has been shown that some proteins, such as tristetraprolin^[24] and AUF1^[25], which also bind to the 3'-UTR, can decrease levels of the COX-2 mRNA. In contrast, other proteins such as HuR, a RNA binding protein prolong the half-life of COX-2 mRNA in colon cancer by binding to the COX-2 AU rich element^[26,27]. High levels of HuR protein have also been reported in HCC cell lines and therefore could be responsible for COX-2 overexpression in this tumor^[28].

As mentioned before, hepatitis C and hepatitis B virus infections are the major etiological agents of chronic liver diseases, which can lead to the development of liver cirrhosis and HCC. However, it is not well known how HBV and HCV are individually involved in human hepatocarcinogenesis. Recent studies have shown that both viruses are able to promote COX-2 expression. After integration of the HBV DNA into the host genome, the expression of the viral protein HBx upregulates COX-2 expression by transactivation of the COX-2 gene promoter through the NF-AT transcription factor^[29,30]. This study therefore demonstrated that COX-2 might be an important cellular effector of HBx protein, which is often the only viral protein expressed by transformed hepatocytes in HCC caused by HBV infection. In addition, the endoplasmic reticulum stress response, due to the expression of the HBV surface protein, may also lead to COX-2 expression through the activation of NF-κB and p38 MAPK^[31]. Similarly, a recent study showed that infection with HCV induces the production of ROS and subsequent activation of NF-κB, which in turn mediates COX-2 expression and subsequent PGE₂ production^[32]. These studies, therefore, provide new insights into the mechanisms by which hepatitis viral infection, through increasing COX-2 expression and PGs production, might be relevant to the development of liver diseases and hepatocarcinogenesis.

It has been suggested that there is another COX enzyme formed as a splice variant of COX-1^[33], referred to as COX-3. COX-3 is made from the COX-1 gene but retains intron 1 in its mRNA. Its expression was initially reported in the canine cerebral cortex and in lesser amounts in other analyzed tissues^[33]. Recent molecular biology studies revealed that indeed three distinct COX-1 splicing variants exist in human tissues^[34]. The most prevalent of these variants, called COX-1b1, arises via retention of the entire intron 1, leading to a shift in the reading frame and premature termination. This would make the expression of a full-length protein impossible, therefore a catalytically active form of the enzyme might

Brand	Celebrex	Bextra	Vioxx	Arcoxia	Prexige
Generic:	Celecoxib	Valdecoxib	Rofecoxib	Etoricoxib	Lumiracoxib
Chemistry:	Sulphonamide	Sulphonamide	Sulphonyl	Sulphonyl	Phenylacetic acid
COX-1/COX-2 ratio					
Pharmacokinetics:					
Oral bioavailability (%)	22-40	83	92-93	100	74
Tmax (h)	2-4	2.3	2-3	1	2-3
Half-life (h)	11	8-11	10-17	22	3-6
Vol. Dist. (L)	455	86	86-91	120	9
Plasma protein binding (%)	97	98	87	92	> 98
Metabolism					
Main pathway	Oxydation CyP450 (2C9, 3A4)	Oxydation CyP450 (2C9, 3A4)	Cytosolic reduction	Oxydation CyP450 (3A4)	Oxydation CyP450 (2C9)
Urinary excretion (%)	29	70	72	60	54

Figure 2 Pharmacological features of coxibs.

not exist in humans. However, the other two variant types, called COX-1b2 and COX-1b3, although retaining the entire intron 1, lack a nucleotide in one of two different positions, thereby encoding predicted full-length and probably COX-active proteins, as suggested by functional studies, which revealed that COX-1b2 is able to catalyse the synthesis of PGF₂α from AA^[34].

COX INHIBITORS

NSAIDs have long been known as drugs that have the three favorable analgesic, anti-pyretic and anti-inflammatory effects. However, NSAIDs differ in their therapeutic potency, gastrointestinal side effects and COX inhibition ratios. NSAIDs cover a wide range in their ratios of inhibitory potencies (i.e. selectivity) towards COX-1 and COX-2. Some NSAIDs have moderate selectivity for COX-1 (e.g., ketorolac, flurbiprofen, ketoprofen, piroxicam), others inhibit both COX isoforms (dual inhibitors; e.g. indomethacin, aspirin, naproxen, ibuprofen), other NSAIDs favor COX-2 inhibition (e.g. sulindac, nimesulide, etodolac, meloxicam), and finally the newest ones are highly selective for COX-2 (COXIB; e.g. celecoxib, rofecoxib, lumiracoxib, valdecoxib, etoricoxib) (Figure 2). Although the mechanism of action of the different COXIB is similar, their chemical structures differ. In addition, the pharmacokinetics and metabolism of each individual COXIB are unique (Figure 2)^[35,36].

COX IN HEPATOCELLULAR CARCINOMA

Strong support for a connection between COX-2 expression and carcinogenesis has come from genetic studies. The number and size of intestinal polyps in APC^{Δ716} mice, a murine model of human familial adenomatous polyposis coli (FAP), were reduced in animals that were engineered to be also COX-2 deficient^[37]. In a separate study, homozygous deficiency of COX-2 reduced skin tumorigenesis in a multistage mouse skin model^[38]. On the contrary, overexpression of COX-2 was sufficient to induce tumorigenesis in transgenic mice^[39-41].

The evidence that COX-2 may be a logical therapeutic target in HCC comes from studies that showed overexpression of COX-2 in patients with HCC^[42-46]. COX-2 expression is generally higher in well-differentiated HCCs compared with less-differentiated HCCs or histologically normal liver, suggesting that COX-2 may be involved in the early stages of hepatocarcinogenesis^[42,44,46]. In addition, a significant correlation between COX-2 expression and active inflammation in the adjacent noncancerous liver has been reported^[43,47], and increased expression of COX-2 in noncancerous liver tissue was significantly associated with shorter disease-free survival in patients with HCC^[43]. This result is of great importance from a clinical point of view, as it suggests that COX-2 expression may play an important role in the relapse of HCC after surgery.

Furthermore, we recently reported that COX-2 expression in the tumor tissue was significantly correlated to the presence of inflammatory cells, macrophages and mast cells^[46]. However, COX-2 expressing cells and the number of both types of inflammatory cells decreased with progression of the disease, suggesting their possible involvement in the early stages of hepatocarcinogenesis.

The decrease in COX-2 expression during tumor progression as observed in HCC is unusual. A possible explanation for this different behavior pattern is that, in some cell types, COX-2 overexpression may cause a growth disadvantage, as suggested by Trifan^[48], who reported that COX-2 overexpression may induce cell cycle arrest in a variety of cell types.

Although less attention has been drawn to the potential role of the constitutive COX-1 enzyme in carcinogenesis, recent evidence supports its implication in skin and intestinal tumorigenesis^[38,49-52]. COX-1 is up-regulated in human breast^[53], prostate^[54], cervical^[55] and ovarian cancers^[56,57]. On the other hand, loss of the COX-1 gene results in reduced intestinal tumorigenesis in Min mice^[49].

We recently analyzed COX-1 expression in HCC and the surrounding non-tumor tissues^[58]. On the whole, we found a higher COX-1 expression in the cirrhotic liver

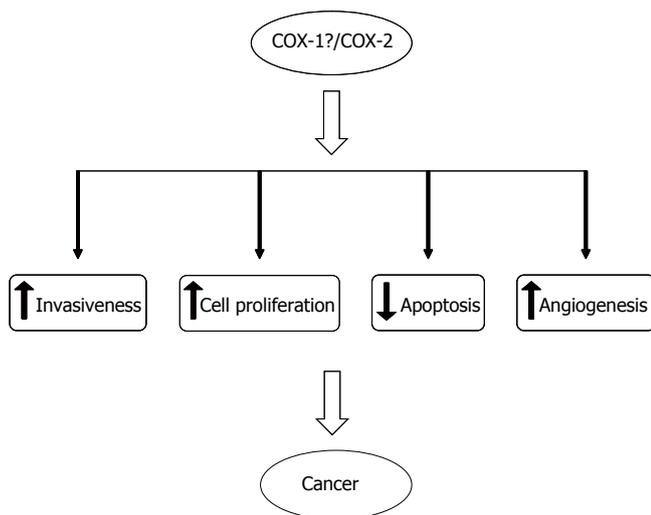


Figure 3 Effects of COX enzymes on different cellular dynamics.

tissues surrounding HCC than in the tumors. However, in some cases COX-1 was up-regulated in the tumor tissues compared to the adjacent non-tumoral cirrhotic tissues. In well-differentiated HCC, COX-1 expression was significantly higher than in the poorly-differentiated tissues, suggesting that the presence of COX-1 might be also involved in the early stages of tumor growth.

COX INHIBITORS IN HEPATOCELLULAR CARCINOMA

Evidence from animal models

Experimental studies on animal models of liver cancer have shown that NSAIDs, including both selective and non-selective COX-2 inhibitors, exert chemopreventive as well as therapeutic effects^[59-64]. In the rat model of choline-deficient, L-amino acid-defined diet (CDAA)-induced hepatocarcinogenesis the administration of aspirin or nimesulide with the diet decreased the number of preneoplastic and neoplastic nodules^[60,63]. In a recent study by Marquez-Rosado^[64] treatment with celecoxib was highly effective in inhibiting the multiplicity and size of liver preneoplastic lesions induced by DEN, 2-AAF and partial hepatectomy.

The therapeutic potential of the specific COX-2 inhibitors, such as celecoxib and meloxicam, in HCC generated in nude mice has also been shown^[65,66]. The treatment significantly reduced the growth of HCC *in vivo* by enhancing tumor cell apoptosis and reducing proliferation.

Overall, these results suggest that NSAIDs and other selective COX-2 inhibitors may be of value in the chemopreventive as well as therapeutic activities against liver cancer.

Evidence from "in vitro" experiments

The involvement of COX-2 in carcinogenesis is believed to be primarily mediated through its influence on cell proliferation, apoptosis, angiogenesis and cell invasiveness^[67] (Figure 3).

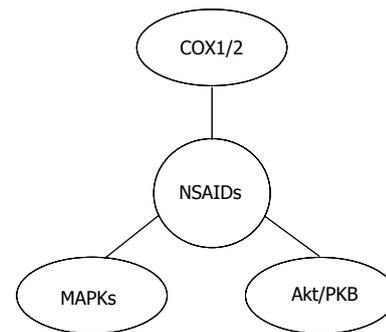


Figure 4 Molecular targets of NSAIDs in HCC.

The role of COX-2 in the stimulation of cell proliferation can be attributed to its involvement in the production of prostaglandins. Indeed, evidence indicates that PGs promote cell proliferation, and conversely the growth-inhibitory effects of COX inhibitors can be reversed by exogenous addition of PGs. It has been demonstrated that prostaglandins increase DNA synthesis and cell proliferation of rat hepatocytes^[68,69], and of human HCC cells^[45].

On the other hand, it has been demonstrated that COX-2 inhibitors are able to suppress HCC cell growth^[44,45,58,70-74]. Several mechanisms have been proposed for the antitumor effects of NSAIDs in HCC. However, the key mechanism by which COX-2 inhibitors affect HCC cell growth remains unclear. Some studies have shown that NSAIDs are able to inhibit HCC cell growth by cell cycle arrest^[72,73,75], induction of apoptosis^[44,73,74] or necrosis^[72].

Recent evidence indicates that pharmacological inhibition of COX-1 activity by selective COX-1 inhibitors also blocks cell growth, promotes apoptosis and inhibits the cell cycle in ovarian^[57], breast^[76], bladder and prostate^[77] cancer cells. In addition, a combination of COX-1 and COX-2 selective inhibitors was found to suppress polyp formation more effectively in the intestinal tumorigenesis of the *Apc* knockout mouse model^[52]. Interestingly, we recently showed that the selective COX-1 inhibitor SC-560 inhibits cell growth and induces apoptosis in HCC cells^[58]. Moreover, the combination of the COX-1 inhibitor with selective COX-2 inhibitors, resulted in additive effects on cell growth inhibition. These results suggest that both COX-1 and COX-2 inhibitors may have potential therapeutic implications in HCC patients.

However, it is still controversial whether the antitumor effects of COX-2 inhibitors in HCC are due predominantly to the inhibition of COX-2 activity^[45,58]. Indeed, the antineoplastic effect of NSAIDs might not be mediated only by COX-2 inhibition, but NSAIDs might act on different molecular targets as well^[78].

Increasing evidence suggests the involvement of molecular targets other than COX in the antitumor effects of selective inhibitors also in HCC, including the mitogen-activated protein kinase (MAPK)^[79] and the PI3K/Akt pathway^[45,70] (Figure 4). The existence of COX-independent mechanisms of NSAIDs action is further supported by the evidence that their antineoplastic effects are observed with concentrations that are greater than

those necessary to fully inhibit the synthesis of PGs, and by the observation that they inhibit HCC cell proliferation in COX-2 negative cells^[79]. Interestingly, COX-2-independent effects of celecoxib have also been observed during hepatocarcinogenesis *in vivo*. In the study by Marquez-Rosado^[64] neither COX-2 expression nor PGE₂ production were altered by celecoxib treatment, suggesting that celecoxib effects are mediated by COX-2/PGE₂-independent mechanisms.

COX-2 AND HCC ANGIOGENESIS

A substantial body of evidence supports a role for COX-2 in angiogenesis, the “sprouting” of capillaries from pre-existing vasculature, in a variety of human malignancies^[80-83]. COX-2 promotes angiogenesis, mainly through the synthesis of prostanoids, which can induce tumor angiogenesis in an autocrine and/or paracrine fashion by stimulating the expression of pro-angiogenic factors^[84,85]. However, the precise role of each individual prostanoid remains largely unknown.

COX-2 expression has been reported to correlate with tumor angiogenesis in patients with HCV- or HBV-associated HCC^[86,87]. Moreover, in a recent study we showed a positive correlation between COX-2 expression in tumor tissues of HCC patients and the presence of microvessels inside the tumor mass, assessed by staining endothelial cells with anti-CD34 antibody^[46]. In addition, we reported that COX-2 was the only independent variable that showed a positive correlation with CD34 in a multivariate analysis, confirming the possible role of COX-2 in HCC angiogenesis. These findings suggest the hypothesis that selective inhibition of COX-2 by treatment with COXIB may contribute to inhibit HCC-associated angiogenesis, and thus provide an additional rational approach for treatment of this malignancy.

COX-2 AND INVASIVENESS OF HCC CELLS

A link between COX-2 expression and invasiveness has been observed in several human malignancies^[88,89]. Colon cancer cells that constitutively expressed COX-2 acquired increased metastatic potential that could be reversed by treatment with COX inhibitors^[90]. This phenotypic change was associated with increased expression and activation of metalloproteinase-2 (MMP-2)^[90]. Similarly, PGE₂ induces MMP-2 expression and activation in HCC cells^[91], and treatment with aspirin and with the selective COX-2 inhibitor NS-398 inhibits the HGF-induced invasiveness of HCC cells^[92], suggesting the key role of the COX-2/PGE₂ pathway in tumor invasiveness of liver cancer.

COX-2 AND MULTIDRUG RESISTANCE

Growing evidence indicates that COX-2 overexpression can up-regulate the expression of the Multidrug Resistance 1 (MDR1) gene and the levels of its product, the multidrug efflux pump P-glycoprotein (P-gp)^[93,94]. COX-2 could therefore contribute to the development of resistance

to pharmacological treatment by the tumor cells^[93,94]. Recently, the MDR phenotype was associated with COX-2 overexpression in liver cancer cells^[95].

It could be speculated that a selective inhibition of COX-2 activity could reinforce the antitumor action of conventional chemotherapy by acting on the expression of P-gp. The rationale behind the possible combination of traditional chemotherapy and selective COX-2 inhibitors is further supported by the fact that chemotherapy itself induces COX-2 expression^[96].

CONCLUSION

There is compelling evidence that COX-2, and also COX-1, have a role in hepatocarcinogenesis, but many questions need to be answered. A number of studies have shown that several different mechanisms may account for the anticancer effects of NSAIDs, although the main mechanism remains unclear. The effects of NSAIDs on tumor growth are most likely to be multifactorial, and COX-inhibitors may use both COX-2 and non-COX-2 targets to mediate their anti-HCC activities. Consequently, a better understanding of the COX-2-dependent and COX-2-independent pathways may help to optimize the use of COX-2 inhibitors in the prevention and treatment of HCC.

Recently, concern was raised about the cardiovascular safety of the selective COX-2 inhibitor Rofecoxib^[97,98], and as a consequence it was withdrawn from the USA market by Merck and Co. Further investigation is required to define the safety profile of selective COX-2 inhibitors, especially when they are used at high doses and for long periods of time.

An exciting, novel concept in cancer chemoprevention and treatment is the use of a combination therapy. A combination therapy (which may allow dose reduction, and hence decreased systemic bioavailability) of NSAIDs or COXIBs with agents that specifically modulate relevant biochemical targets of COX-2 inhibitors may take advantage of synergistic growth inhibitory effects against cancer cells and could reduce the toxicity associated with the intake of COX-2 inhibitors. In addition, the use of COX-2 inhibitors, by their action on the MDR phenotype, may enhance the accumulation of chemotherapy agents and decrease the resistance of tumors to chemotherapeutic drugs. Indeed, several clinical trials are under way based on combinations of COXIBs with conventional anticancer treatments (chemotherapy or radiotherapy)^[99] and with novel molecular targeting compounds^[100].

On the other hand, since experimental studies have provided evidence that PGs are the molecules that mediate the effects of COX overexpression, other molecules involved in PG biosynthesis and signaling might represent potential targets. Recently, pharmacological inhibitors of PGE₂-EP receptors, which have anti-neoplastic activity, have been generated^[101]. Therefore, PG receptors and/or PG synthases may represent novel targets for the prevention and treatment of cancer.

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REVIEW

Transfusion transmitted virus: A review on its molecular characteristics and role in medicine

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Abstract

The present review gives an updated overview of transfusion transmitted virus (TTV), a novel agent, in relation to its molecular characteristics, epidemiological features, modes of transmission, tissue tropism, pathogenesis, role in various diseases and its eradication from the body. TTV, a DNA virus, is a single stranded, non-enveloped, 3.8 kb long DNA virus with a small and covalently closed circular genome comprising 3852 bases. It was tentatively designated *Circinoviridae* virus. TTV genome sequence is heterogeneous and reveals the existence of six different genotypes and several subtypes. TTV has been reported to transmit not only *via* parenteral routes, but also *via* alternate routes. This virus has been detected in different non-human primates as well. At present, TTV is detected by polymerase chain reaction (PCR) with no other available diagnostic assays. It shows its presence globally and was detected in high percent populations of healthy persons as well as in various disease groups. Initially it was supposed to have strong association with liver disease; however, there is little evidence to show its liver tropism and contribution in causing liver diseases. It shows high prevalence in hemodialysis patients, pointing towards its significance in renal diseases. In addition, TTV is associated with several infectious and non-infectious diseases. Although its exact pathogenesis is not yet clear, TTV virus possibly resides and multiplies in bone marrow cells and peripheral blood mononuclear cells (PBMCs). Recently, attempts have been made to eradicate this virus with interferon treatment. More information is still needed to extricate various mysteries related to TTV.

INTRODUCTION

Transfusion transmitted virus (TTV) is a recently discovered virus which was suspected to be a causative agent of non-A to non-E hepatitis. TTV was first identified in the serum of a patient who was hospitalized with post transfusion hepatitis of unknown etiology in 1997^[1]. Initially, TTV was described as a non-enveloped, 3739 bases long and single stranded DNA virus. Based on its genomic characteristics, it was reported to be a parvovirus-like pathogen. Later, studies on the molecular and biophysical characteristics of TTV demonstrated this novel agent as a non-enveloped virus with a small, covalently closed circular genome of single stranded DNA comprising 3852 bases^[2-4]. Its buoyant density is significantly different from that of parvoviruses. Also there was a significant sequence difference between TTV and members of the *Circoviridae*^[5]. It was proposed that TTV belongs to a new virus family that was tentatively designated *Circinoviridae*^[2] *paracircoviridae*^[6] or the TTV family^[7] by different research groups. TTV isolates have an extremely wide range of sequence divergence^[8-10] and were tentatively classified into 23 genotypes with sequence divergence of > 30% from one another^[11] or into four major phylogenetic groups^[7,11]. TTV like mini virus (TLMV) with 2.8-3.0 kb genomic length was also identified in humans and chimpanzees^[12-14].

Since the discovery of TTV, studies have been published describing the prevalence of TTV infection in people with acute or chronic hepatitis as well as in blood donors and drug users and also in healthy persons^[15-17]. It is apparent that currently it is not possible to ascribe TTV to any specific diseases. TTV can be transmitted parenterally and has been found in plasma and peripheral blood mononuclear cells. However, non-parenteral transmission is also possible as TTV can be excreted in feces^[10]. Molecular and phylogenetic analysis of polymerase

chain reaction (PCR) fragments revealed that TTV could be divided into several genotypes that are found worldwide without any direct correlation with geographical distribution of diseases^[2, 18-20]. This is an interesting area to investigate different aspects of TTV and several groups are working to extricate many mysteries related to this agent world over. Until now, an abundance of information has been published on TTV in relation to its molecular form and infectious status.

To have a compilation of information and also have a better understanding of TTV, the present article provides a holistic view on various characteristics of this novel agent with particular emphasis on its molecular characteristics, epidemiological features, endemic behavior and pathogenesis as well as prospects of its eradication on the basis of published information.

MOLECULAR BIOLOGY

TTV, a common virus in humans with high prevalence in the general population^[21,22] is a single stranded DNA virus. Its the genome was sequenced by Okamoto *et al*^[3] almost entirely on the prototype isolate TA278 encompassing 3739 nucleotides and was temporarily believed to be a linear DNA. However, later studies^[2] with the GHI isolate and TA 278 isolate^[4] have identified a GC-rich missing link of about 100 nucleotides that complete the TTV genome as a closed circular DNA with a length of 3852 nucleotides (nt) and a particle size of 30-50 nm. Thus, TTV is an unenveloped virus whose genome consists of a circular and single stranded DNA molecule of negative polarity and about 3.8 kb length^[2]. TTV has an isopycnic density of 1.31-1.34 g/mL in CsCl^[2,4]. The TTV genome has two or three possible open reading frames (ORFs) capable of encoding 770 aa (ORF1), 202 aa (ORF2) and 105 aa (ORF3) polypeptides^[4]. Analysis of the TTV transcription pattern in COS-1^[23] and bone marrow cells^[24] has revealed the existence of at least three species of spliced mRNA molecules of 2.9-3, 1.2, and 1.0 kb in length, with common 5' and 3' termini, leading to the creation of new reading frames (ORF3 and ORF4) in addition to the previously described ORF1 and ORF2^[25].

Many studies have indicated a high degree of genetic diversity of TTV. The entire genome was sequenced for SANBAN and TA 278 isolates^[26]. The genetic organization of the genome was similar in two isolates: two open reading frames (ORF1 and ORF2) were sandwiched by the motifs of TATA box and polyadenylation signal, and a GC-rich short stretch resided at the midst of the untranslated region. No other ORFs longer than 300 nt and common to SANBAN and TA278 were found. The overall nucleotide sequence identity between the two isolates was 56.7%, significantly lower than that (93%) between TA278 and GH1^[2]. Interestingly, the nucleotide sequence identity was relatively higher in the untranslated region (73.0%) than in the translated region (52.2%). A great degree of genetic diversity for a group of viruses represented by TTV most likely had a long history of evaluation and adaptation to humans. Despite the extensive sequence divergence of TTV and TLMV in coding regions of the genome, three areas of remarkable conservation

have been identified in the part of UTR that contains promoters and splice sites^[24]. Sequence conservation is found among all known human isolates of TTV and TLMV as well as those recovered from a range of non-human primates.

Based on its physico-chemical and genomic characteristics^[2-4], TTV was proposed as a member of new viral family tentatively named the *Circoviridae*^[3]. Although TTV shares some features, such as a negative-stranded circular DNA genome, with members of the *Circoviridae*^[1], the genetic organization of its genome is distinctly different from that of viruses that belong to this family. Therefore, TTV was tentatively classified into the virus family, *Circoviridae*^[3].

GENOTYPES

The TTV genome sequence is extremely heterogeneous. Phylogenetic analysis performed on TTV isolates recovered from several parts of the world revealed the existence of 6 different genotypes^[3,27]. The sequence heterogeneity of the TTV genome, however, is more complex. One report described the identification of 16 TTV genotypes^[28]. Another study identified 5 additional TTV genetic groups^[10]. One of these was found to represent an additional TTV genotype, whereas the other 4 additional genetic groups were significantly distinct from TTV and from one another compared to the original TTV genotypes. This observation suggested that these 4 new genetic groups represent closely related, yet different, TTV-like viral species. The existence of genetic divergence between different TTV isolates beyond genotypes was noted by another group of researchers^[8]. It has been hypothesized that a whole "swarm" of numerous TTV-like species circulates in the human population worldwide^[29]. Despite this extensive sequence diversity, all variants of TTV share a common genomic organization with three predicted encoded proteins of similar length and likely function.

TTV was originally found in humans; however, recent studies showed that TTV can also be identified in serum specimens obtained from domesticated farm animals^[29] and from non-human primates^[29,30]. Phylogenetic analysis using the TTV sequence obtained from animals demonstrated that these sequences belong to already known human TTV genotypes^[29,30], although some sequences recovered from nonhuman primates remain unclassified^[30]. The results of these experiments demonstrated that chimpanzees may be infected with some TTV-related species that have not been found in humans. An additional phylogenetic analysis using all known TTV sequences, some of which were not classified^[26,30] or were classified previously as new TTV genotypes^[10], suggested the existence of 16 genotypes or 13 different TTV-like species. The prototype TTV-I^[8] sequences were classified into eight genotypes found in humans as well as in non-human primates and farm animals. Moreover, genetically distant variants, namely PMV, SANBAN and SEN viruses have been identified. Frequent homologous recombination, which can occur when a subject is co-infected with two or more isolates, is an important multiplier of the TTV genetic diversity. This

phylogenetic tree contains 13 major groups. Each major group represents sequences that are more distant from the other major group sequences. This observation is strongly supported by the analysis of frequency distribution of evolutionary distances. Comparison of sequences from one major group of branches to all other branches demonstrates that each of the 13 major groups of branches may represent different viral species. All of these viruses are closely related to the prototype TTV strains tentatively designated TTV-I. The other viral species identified with each major branch were also designated TTV with the addition of Roman numerals (e.g. TTV-II, TTV-III) as proposed previously^[8].

All 13 major groups of branches or viral species may be arranged into four groups. Group A contains viral species I and IX; group B contains II, III, X, XI, XII, and XIII; group C contains IV and V; and group D contains VI, VII, and VIII. The TT viruses found in humans belong to groups A, B, and C. Group C is composed exclusively of viruses found in humans and group D of viruses found only in non-human primates. The primate TT viruses can be also found in groups A and B. Group A includes two viral species. One of these viruses is the prototype TTV-I, which was originally identified in a Japanese patient with parenterally transmitted hepatitis of unknown etiology^[1]. Previously, seven TTV-I genotypes were identified^[27,28]. However, based on phylogenetic analysis, TTV-I genotypes 2 and 3 were suggested to be combined as genotype 2/3^[8]. This suggestion reduces the number of TTV-I genotypes to 6. Earlier, several new TTV variants were found in non-human primates^[29,30]. The phylogenetic analysis performed in this study confirmed that sequences Bo-Ho and Bo-De identified by Vergchoor *et al* belonged to genotype 2/3. However, sequences Ch-Pe and Ch-Br2 constituted two new TTV-1 genotypes^[29] whose sequences constituted a new major branch in the phylogenetic tree representing TTV-IX, which is different from, but closely related to the prototype TTV-I. TTV-IX detected in chimpanzees is most closely related to the TTV-I genotype 8 found only in non-human primates^[30]. Recently, several new TTV variants were identified in serum specimens from healthy Japanese individuals^[31]. Group B contains TTV-II, TTV-III, TTV-X, TTV-XI, TTV-XII and TTV-XIII, which can be found in non-human primates as well as in human specimens. TTV-II, TTV-III, TTV-X and TTV-XIII were found only in humans. Group C is composed of two human viruses, TTV-IV and TTV-V. Group D consists of three new chimpanzee viruses, namely TTV-VI, TTV-VII and TTV-VIII.

HUMAN TRANSMISSION

TTV was first characterized as a blood-borne virus and was thus referred to as a transfusion-transmitted virus (TTV)^[3,32]. However, later studies suggested the existence of other routes of transmission also. In fact the mechanism of TTV transmission has not yet been elucidated. The higher prevalence of TTV in persons treated with blood^[3] or blood products^[32] has suggested parenteral transmission as a frequent route of TTV infection. TTV is common in patients who have an increased risk of infection with blood-borne viruses, such as hemophiliacs (68%), patients

on maintenance hemodialysis (46%), and abusers of intravenous drugs (40%)^[31]. There is a high prevalence of TTV in blood products. TTV contamination was found in 10 of 18 batches (56%) of factor VIII and IX concentrates manufactured from non-remunerated donors, and in 7 (44%) of 16 batches of commercially available products^[32]. These observations suggest that TTV is transmitted by blood and blood products. However, infrequent detection of TTV-DNA in serum samples from prostitutes and homosexual men^[17] and the findings of fecal^[33] and bile^[34] excretion of TTV indicate that TTV may have characteristics different from other blood-borne viruses. The prevalence of TTV in blood donors in different countries varies between 1.9% to 62%^[3,22,27,32,35]: 1.9% in England^[32], 9.1%-12.8% in United States^[27], 12% in Japan^[3], 36% in Thailand^[35] and 62% in Brazil^[22]. Moreover, the majority of TTV infected people had no history of blood and/or blood products transfusion. The relatively high prevalence of TTV in blood donors and the large proportion of TTV infected patients with no history of transfusion of blood and blood products also suggest that alternative routes of transmission of TTV infection may exist^[36]. TTV DNA has also been detected in saliva^[36], throat swabs^[37], breast milk^[38], semen^[39] and vaginal fluid^[40] thus, supporting routes of transmission other than blood and blood products^[33,37].

Excretion of TTV in feces of infected individuals suggests of possible fecal-oral transmission^[33]. Some studies have reported placental transmission of TTV^[41-43], whereas others have not detected TTV in cord blood and amniotic fluid^[44-45]. These studies show the absence of transuterine transmission of TTV. Since children of TTV-infected mothers apparently tend to get infected more often and earlier after birth than children of TTV negative mothers, the role of postnatal transmission of TTV is being considered. Postnatal route of transmission from mother to child and infection *via* frequent social contacts seem to be very important modes of transmission in children^[46-48]. Furthermore, variation in the TTV prevalence in children from 5.1% in Japan^[49] to 54% in the Democratic Republic of Congo^[50] is also suggestive of the possible involvement of some specific environmental factors in the acquisition of TTV infection. The sexual mode of transmission is likely of low effectiveness^[51].

ANIMAL TRANSMISSION

Infection with both TTV and TLMV has been detected in various non-human primates^[2,52,53]. At the same time, there are reports showing cross-species transmission of TTV genotypes. Human TTV variants can infect chimpanzees and macaques^[13,54]. Beyond primates, host range of TTV and TLMV is uncertain. One study has demonstrated frequent TTV infection of domestic animals such as cows, pigs, sheep and chickens^[47]. However, it is not known how these species acquire TTV infection. Recently, highly divergent TTV like viruses were detected in pigs, cats and dogs, distinct from those found using the N22 primers^[55] suggesting that this virus family may indeed be widely distributed in the mammalian order. There are reports showing high prevalence of TTV infection in captured

chimpanzees and crab eating macaques^[56]. These findings suggest that TTV is widespread among wild Chimpanzees living in West Africa. However, this TTV infection was found non pathogenic. Based on analysis of full-length sequence data, this TTV may represent a new TTV-like viral species or genus, although it is closely related to human TTV^[56].

TARGET ORGANS

Regarding target organs for TTV infection, TTV-DNA has been detected by both PCR and in situ hybridization in liver and peripheral blood mononuclear cells (PBMC)^[57,58]. However, these studies have shown that TTV replicates in liver, but not in PBMC. Simultaneously, TTV was detected and found with replicative intermediates in bone marrow cells from TTV infected patients^[24]. This finding and few other studies^[59] indicate that TTV-DNA in PBMC corresponds to viral particles passively attached to cell membrane and TTV infects hematopoietic cells but only replicate when these cells are activated. Since the percentage of circulating activated cells is very low, this may be the cause for the lack of detection of TTV replicative intermediates in freshly isolated PBMC from TTV infected patients.

DIAGNOSTIC ASSAYS

The development of sensitive and reliable polymerase chain reaction (PCR) protocols allowed the detection of TTV DNA at a very high prevalence in sera of healthy populations around the world^[21,22,29]. Currently, the heteroduplex mobility assay to detect multiple infections with isolates of TTV belonging to different genotypes or subtypes has also been developed. In the simplest application of heteroduplex mobility assay, heteroduplexes are formed by denaturing and reannealing mixtures of PCR amplified DNA fragments from divergent isolates of the same virus. When these products are separated on polyacrylamide gels a homoduplex band plus two slow moving heteroduplex bands are observed. The mobility of heteroduplexes is related to the genetic distance between two strands. This technique was applied earlier to HIV isolates, measles virus, CMV and hepatitis C virus. While detecting TTV DNA by PCR, it was found that repeated freezing and thawing of serum did not have much effect on stability of TTV DNA. Specimens are not required to be aliquoted for repeated testing and retrospective studies^[60].

Both TTV and TLMV have sequence heterogeneity. Some TTV subtypes have less than 50% sequence identity. However, there are certain conserved regions. Primers were designed in such a way that most of the subtypes could be detected^[61]. Recently, real time PCR based methods with either SYBR Green or TaqMan Probe, designed to quantitate selectively TTV and TLMV, have also been used^[61].

EPIDEMIOLOGY

Epidemiological studies have shown that TTV is described

worldwide in various populations. The prevalence of TTV viremia in healthy adults of developed countries is in the range of 1%-34%. Prevalence reported from third world countries was found to be higher, typically 40%-70%. In people who have received multiple blood transfusions the virus is almost universally present with more than one subtype in each individual. Table 1 demonstrates the countrywide prevalence of TTV infection in different categories of populations including both healthy persons and patients with various types of diseases^[62-75].

TTV INFECTION IN LIVER DISEASES

From preliminary reports two characteristics of TTV infection have emerged rendering it as a potential cause of liver disease. First, Okamoto *et al*^[3] demonstrated that TTV-DNA levels in liver tissue were equal to or 10-100 times higher than those in serum, suggesting that this virus replicated in the liver. Second, Nishizawa *et al*^[11] reported the appearance of TTV-DNA in the sera of patients with post transfusion hepatitis of unknown etiology to display close correlation with ALT levels. Neither one of these characteristics, hepatotropism or correlation of viral titres with serum ALT, had previously been demonstrated for HGV. However, most subsequent investigations could not confirm their significance in the development of fulminant hepatitis, cryptogenic chronic liver disease and HCC. In addition, the implications of coinfection with TTV in the natural history of chronic HBV or HCV infection are also far from clear. Although TTV can be transmitted by parenteral route, its role in causing posttransfusion hepatitis has not been established^[76-79]. The majority of individuals who become TTV-DNA-positive after blood transfusion usually have normal ALT and do not develop chronic hepatitis, although TTV viremia frequently persists for several years. Patients who develop chronic hepatitis are invariably coinfecting with HBV or HCV and chronic hepatitis is closely correlated with HBV or HCV infection. This raises the possibility that TTV is merely an innocent bystander rather than a primary hepatitis virus.

In one of the studies^[79], the rate of TTV infections was found to be significantly higher among transfused than among non-transfused patients (26.4% and 4.7%, respectively) and the risk of infection increased with the number of units transfused. The rate of TTV infections with non A-E hepatitis (23.2%) was almost identical to the rate among patients who had been transfused, but did not develop hepatitis (21.8%). Of those patients with acute hepatitis C, 40.0% were simultaneously infected with TTV and TTV did not worsen either biochemical severity or persistence of hepatitis C. In non-A-E cases, the mean ALT was comparable among those positive for TTV and those negative. Neither was there a consistent relationship between ALT and TTV-DNA level among these patients^[80].

The role of TTV in acute hepatitis is another unresolved issue. In two Japanese studies^[81,82] TTV-DNA was identified in 13.6%-43% of cases of non-A-E community-acquired acute hepatitis. However, these positive rates of TTV do not differ statistically from either those obtained among patients with other types of viral

Table 1 Global prevalence of TTV infection in normal subjects and patient populations

S.No.	Country	Group	No. tested	No. Positive (%)	Reference No.
1	Italy	Patients with different clinical diagnosis			62
		Unselected pathologies	221	110 (50)	
		Hemophilia A	33	24 (73)	
		Hemodialysis	36	19 (53)	
		HCV positive patients			
		Normal ALT	30	17 (57)	
		Abnormal ALT	50	24 (48)	
		Cirrhosis	30	9 (30)	
		HCC	13	8 (62)	
		HCV negative patients			
		Non-A non G hepatitis	23	11 (48)	
		Autoimmune hepatitis	11	4 (36)	
		Primary liver diseases	17	11 (65)	
		Cryptogenic extrahepatic diseases			
		Systemic lupus erythematosus (SLE)	34	19 (56)	
		Psoriasis	102	56 (55)	
		Rheumatoid arthritis	60	17 (28)	
2	Italy	Healthy blood donors	100	22 (22)	63
		Hemophiliacs	178	123 (69)	
3	Italy	Patients			64
		HIV I infected mothers	83	29 (34.9)	
		- Intravenous drug users	46	21 (45.6)	
		- Non-intravenous drug users	37	8 (21.6)	
		Uninfected			
		- Infants born to TTV infected mothers	29	8 (27.5)	
4	Italy	HIV Negative			65
		- Blood donors	104	91 (87.5)	
		- Chronic hepatitis C	106	99 (93.4)	
		- Hemodialysis patients	100	100 (100)	
		- Thalassemic patients	36	36 (100)	
		- IVDUs	37	31 (83.8)	
		HIV Positive			
		- IVDUs	102	102 (100)	
		- Homosexuals	58	52 (89.7)	
		- Heterosexuals	50	44 (88.0)	
5	Italy	Haemophiliacs	217	204 (94)	66
6	China	Healthy persons	136	29 (21.3)	67
		Prostitutes	140	46 (32.9)	
7	China	Intravenous drug users	50	14 (28)	68
		- Hemophilics	50	35 (70)	
		- Thalassemics	40	27 (67.5)	
		- Hemodialysis patients	50	13 (26)	
		Household contacts			
		- Spouse	40	3 (7.5)	
		- Non spouse	57	7 (12.3)	
		Acute hepatitis A	52	4 (7.7)	
		Non A-E hepatitis			
		- Acute	12	5 (41.6)	
		- Chronic	9	2 (22.2)	
		- Fulminant	11	5 (45.4)	
		Hepatitis B carriers	200	30 (15)	
		Hepatitis C carriers	100	36 (36)	
		Healthy adults	100	10 (10)	
8	China	Healthy children	122	33 (2.7)	69
		Non A-E hepatitis	19	8 (42.1)	
		- Acute	13	6 (46.1)	
		- Chronic	3	1 (33.3)	
		- Fulminant	3	1 (33.3)	
		Thalassemic children	64	47 (73.4)	

S.No.	Country	Group	No. tested	No. Positive (%)	Reference No.
		- Transfused during cardiac surgery	80	37 (46.3)	
		- Chronic HBV carrier	30	10 (33.3)	
		- Biliary atresia	32	5 (15.6)	
9	USA (Minnesota)	Healthy donors with elevated ALT	99	5 (5)	70
		Healthy donors with normal ALT	146	1 (0.7)	
10	Japan	Patients with chronic liver disease of unknown etiology	69	57 (83)	71
		Volunteer blood donors	50	40 (80)	
11	Tanzania	Rural women	156	115 (74)	72
12	India	Sewage water	63	8 (12.7)	73
13	Brazil	Patients sera	184	48 (26)	74
		Patients saliva	167	49 (46)	
14	Norway	Blood donors	201	180 (98.6)	75

HCC: Hepatocellular carcinoma; IVDUs: Intravenous drug use.

hepatitis or among healthy volunteers. In addition, the ALT levels do not show any difference between TTV-positive and TTV-negative patients. Furthermore, the presence of TTV infection had no apparent effect on the clinical course of patients with hepatitis A, B or C. Thus, according to these studies no correlation appears to exist between TTV infection and the clinical features of sporadic hepatitis. Contrasting another Japanese study^[83], TTV-DNA was detected in 2 out of 7 (29%) patients with acute hepatitis of unknown etiology, but in none of the 4 patients with acute HCV-associated hepatitis. At least half of all cases of fulminant hepatitis are seronegative for hepatitis A-E viruses. TTV has been found in 27%-50% of patients with fulminant hepatitis^[3,16,84], but such patients probably received multiple transfusion before testing and recent TTV infection has not always been established. Therefore, it is unclear whether TTV infection is secondary to transfusion or plays an etiologic role in fulminant hepatitis.

Current data suggest that TTV is not the causative agent of chronic liver disease of unknown etiology and neither does it affect the degree of liver damage when present as a coinfection with HBV or HCV^[16,77,84-86]. According to our previous study^[86], for example, TTV-DNA was detected in 20% of the HBV-positive and 19.5% of the HCV-positive chronic liver disease patients, in 8.3% of seronegative chronic liver disease patients, in 8.3% of seronegative chronic hepatitis/cirrhosis patients and 7% blood donors. Yet, no significant differences between TTV infected and non-infected patients were found as to demographic data, assumed source of infection, biochemical abnormalities, or severity of liver histology. Thus, regarding etiology and progression towards serious chronic liver disease, its contribution seems to be minor if not altogether non-existent. Concerning antiviral therapy, there are no data or treatment of patients who are infected with TTV alone since the role of TTV as a cause of chronic hepatitis has yet to be determined. Studies of patients infected with both HCV and TTV who were treated with interferon showed that the responsiveness to therapy was correlated with HCV alone. In addition, certain genotypes of TTV were quite resistant to interferon although interferon effectively reduced HCV in

the same patients^[87].

Because the prevalence of TTV is high among patients with chronic viral hepatitis and cryptogenic liver disease, a similar situation has been anticipated to persist among patients with HCC. However, the prevalence of TTV in Thai patients with HCC has shown a wide range of divergence, for example, 6%-60% and 5%-50% in cases with HBV and HCV markers respectively, and 1%-67% in cases without HBV and HCV markers^[27,88]. Our study demonstrated the majority of TTV infected HCC to harbor double or triple infections with HBV and/or HCV and the prevalence of TTV infection was comparable to that of healthy volunteers^[89]. Contrasting that, another group reported TTV-DNA to occur more frequently in patients with liver cirrhosis and HCC than in those with chronic hepatitis^[27]. Using a case-control study to compare the prevalence of 174 Italian patients and matched controls, it was demonstrated that individuals infected with TTV did not exhibit an increased relative risk for developing HCC^[90]. Furthermore, it has been demonstrated that the TTV genome is not found integrated into host hepatocyte DNA^[91], the one process that might represent a potential risk factor in the development of HCC.

TTV INFECTION IN RENAL DISEASES

Using the polymerase chain reaction (PCR), epidemiological studies have indicated a worldwide distribution of this virus, with prevalence surveys in the general population reporting values of 12% to 19% in Japan^[3,92], 36% in Thailand^[27], 2% to 10% in European countries^[32,85] and 1% in the USA^[16]. In patients on maintenance hemodialysis (HD), who are at an increased risk of parenterally transmitted hepatitis virus infection, a high prevalence (32%-53%) of TTV infection has been reported^[93,94]. However, the transmission route of the virus is still unknown and the question of any association between duration of HD or previous transfusion and TTV infection is still a matter of controversy^[77,95]. There is also little information about the occupational risk of TTV infection in HD unit workers. By using logistic regression analysis, it was shown that a prior blood transfusion and time on HD were not predictors of the presence of TTV-

DNA, so that TTV may have a transmission route not shared by HBV, HCV or HGV. The possibility of TTV transmission, *via* a nosocomial route in HD units, must be considered^[77]. One of the possible routes of transmission in an HD unit is direct from person to person. For this reason, the healthcare staff in the HD unit was suspected as being at high risk for TTV infection. Although a low risk of TTV infection was suggested in hospital staff, there is little information on HD unit workers^[96]. TTV infection rate was not influenced by age, sex, or mean duration of dialysis^[77,79,97]. Nosocomial transmission may account for TTV infection in some patients on hemodialysis^[98].

TTV-DNA genotype 1 (G1) was found to be the main TTV DNA genotype in hemodialysis patients. The fact that hemodialysis patients are polytransfused makes it likely that they are at risk of multiple exposures. Therefore, it was interesting that a significant number of patients were apparently coinfecting with different strains of TTV. Sequence analysis of clones from two patients with apparent mixed infections showed that TTV strains belonging to two different major genotypes could coexist in a single patient. It was suggested that infection with one TTV type does not protect against infection with another TTV type^[99]. The preliminary data suggested that TTV is transmitted mainly *via* a parenteral route^[3,85]. When TTV infection was studied in hemodialysis patients who were monitored for HCV infection, co-infection was found in 48% at enrollment. The follow-up of the renal transplant patients revealed, that the persistence of single TTV variants over a long period after organ transplant was common. Considering the heterogeneity of TTV isolates, this finding is against frequent infection with different nucleotide sequences and so horizontal spread of certain variants could have been expected. Nevertheless, in the examined patient group, permanent infections, with only single nucleotide changes in the consecutive samples of the same patient could be observed and the TTV variant detected in one patient was usually remotely related to the TTV variants infecting the others with 58%-97% nucleotide sequence identity between the variants. As a highly variable region of the TTV genome, the N22 region is a candidate to carry humoral epitopes on the surface of the virions. Mutations in this region were, however, infrequently detected. The host's immunity is a plausible evolutionary driving force of the development of TTV genotypes and variants, which process affects most probably the hypervariable genomic regions. If so, the iatrogenic immunosuppression of the transplant recipients can contribute to the long persistence of one variant, while novel infections are infrequent^[100].

TTV COINFECTION

Accumulating molecular and clinical evidence indicated that the effects of HIV infection can be modified by coinfection with other viruses^[101-104]. However, limited information is available about the prevalence and possible pathogenic role of TTV in HIV infected patients with or without AIDS in relation to specific risk factors, and about the viral titres of TTV. Puig-Basagoiti *et al*^[105] reported no influence of TTV infection on CD4 T cell counts

and clinical or immune status in HIV-infected patients. In a recent study by Martinez *et al*^[106], no relationship was found between TTV DNA detection and HIV category, CD4 count, HBV and HCV infection or demography features. By contrast, Christensen *et al*^[101] reported correlation between a low CD4 T cell count and high TTV titre in Danish patients with HIV infection as well as a possible prognostic significance of TTV viral load in immunocompromised patients. High TTV viremia levels were found to be associated with decreased survival rates. A significantly higher rate of TTV positivity was noted in HIV-infected patients than in HIV-negative healthy individuals by two distinct PCR methods. Although the detectability of TTV by either of the two PCR features was as in previous studies^[105,106], the relative rate of TTV DNA in the patients studied was found to be associated with the HIV viral load and CD4 cell level as well as the development of AIDS.

A series of recent reports has indicated that viral infections can influence the pattern of autoantibody expression in patients with autoimmune diseases. Neidhart and coworkers^[107] demonstrated differences in the autoantibody pattern of patients with SSc with antibodies to cytomegalovirus. Other investigations showed a significantly lower prevalence of rheumatoid factors in RA patients infected with TTV, in comparison with non-infected patients. Hajeer *et al*^[108] reported a negative correlation between anti-parvovirus B19 antibodies and rheumatoid factors in patients with RA. In several studies, the autoantibody pattern found in patients with SSc was found to be similar^[109-111]. Comparison of the autoantibody patterns in virus infected and non-infected patients with SSc showed that continuing GBV-C or TTV infection or both, have no evident effect on the manifestation of autoantibodies. In conclusion, various reports showed neither a higher prevalence of GBV-C RNA and/or TTV DNA, nor changes in the pattern of expression of autoantibodies in patients with SSc. Therefore, these data provided no evidence for an association between GBV-C and/or TTV infections and SSc.

Not only the clinical significance and the pathogenesis of TTV infection but also the association between TTV infection and raised ALT values have been controversial^[1,3,113]. It was found that the raised ALT values were independently related to TTV viraemia among Taiwanese who were not infected with HBV and HCV. TTV infection seemed to be responsible for raised ALT values and to hint positive hepatopathic effects. Tuveri *et al* suggested that TTV might be implicated in a few cases of acute and chronic non A-non G hepatitis^[113]. However, Nakano *et al* reported that TTV was not the main causative agent of cryptogenic liver disease^[114]. Further efforts at confirming the pathogenicity of hepatocyte damage by TTV are necessary. As for direct correlations between TTV and *H pylori* infection, TTV DNA was detected at a similar rate in patients with and without *H pylori* infection, and *H pylori* infection was detected at a similar rate in patients with and without TTV infection. Similarities of prevalence of TTV between patients with and without infection by *H pylori*, and prevalence of *H pylori* between patients with and without infection by TTV, as well as the discrepancy in

age distributions between prevalence of TTV and *H pylori* in our patients with peptic ulcer disease indicate that no correlation exists between TTV and *H pylori* infection, even though the two agents have similar age distributions in the general population and similar routes of transmission have been suggested for the two agents^[115]. According to one report, the high prevalence of genogroup 1 TT virus infection in patients with laryngeal cancer and its striking co-prevalence with human papilloma virus infection is biologically important in the progression of squamous cell carcinoma of the larynx^[116].

TTV might replicate in the respiratory tract^[117]. Also, although we found no evidence that TTV might be the direct cause of ARD, TTV loads in both nasal swabs and plasma samples were substantially higher in subjects with bronchopneumonia (BP) than in the subjects with milder ARD (laryngitis, bronchitis, and bronchiolitis), suggesting among other possibilities that TTV could be locally or systemically immunosuppressive and aggravate disease induced by other agents^[117]. However, there is no information on this matter except for recent report showing an inverse relationship between TTV burdens and CD4 cell counts in patients with human immunodeficiency virus type^[101,118,119].

PATHOGENESIS

Although liver tropism has been suggested, TTV also has been found in other organs including kidneys, prostate, mammary glands, brain, bone marrow cells (BMCs) and peripheral blood mononuclear cells (PBMCs)^[3,59,120,121]. Although it is not known precisely in which cell(s) TTV replicates, TTV DNA has been detected frequently in the PBMCs^[58,120] and it has also been suggested to infect and replicate in hematopoietic cells in the bone marrow^[122,123]. Earlier reports had revealed that there was a higher TTV genome load in the PBMCs of cancer patients than in healthy controls (blood donors)^[124]. This could have been related to immune abnormality in cancer patients when compared with the controls, thereby allowing increased TTV replication in the former.

It was demonstrated that TTV is present in the nucleus and cytoplasm of some of the PBMCs. It is possible that infection of immune cells could facilitate escape of the virus from the immune response. Concealed as a “Trojan horse”, TTV in PBMCs might serve as a reservoir of TTV for chronicity of the infection and transmission in some clinical and epidemiological settings. The observation that TTV-negative PBMCs bound considerable virus *in vitro* suggests that at least some TTV found associated with *ex vivo* derived PBMCs might be of plasma origin rather than produced by the cells themselves. We have, however, obtained evidence indicating that PHA-stimulated PBMCs support TTV replication *in vitro* and release substantial titres of virus into the culture fluid. TTV-related single-stranded DNA viruses such as circoviruses and parvoviruses require actively multiplying cells for productive replication^[125,126]. That proliferating hematopoietic cells might be an important source for the TTV that circulates in infected individuals is suggested by findings showing that baseline TTV viremia decreased

markedly in virus-positive prospective bone marrow transplant recipients after myelosuppression with cyclophosphamide and total body irradiation^[122]. Whether TTV is also dependent on cell cycling for active replication remains to be formally established.

It is well known that some viruses can be activated by immune stimulation, most notably HIV, which requires lymphocyte activation for optimum replication^[127,128]. In this regard, it has been shown recently that an animal circovirus related to TTV, porcine circovirus type 2 (PCV-2), can replicate to higher levels in piglets as a result of immunization with a bacterial porcine vaccine^[129]. One major distinction between PCV-2 stimulated in this study and TTV is that the porcine virus is capable of causing a wasting syndrome and may do so more effectively following immune stimulation. In addition, immune suppression due to drugs or induced stress resulted in a transient increase in TTV titers in humans^[130,131]. This indicated that resting PBMCs could not produce TTV, but that mitogen activated cells could be stimulated *in vitro* to replicate TTV. Thus, there is a suggestion that TTV levels may be related to the state of activation of the host immune system as postulated in an earlier publication^[132].

Recently, replicative circular double-stranded intermediates of TTV DNA have been reported in liver and BMC^[24,133]. TTV DNA has been detected by *in situ* hybridization in nuclei of hepatocytes from experimentally infected rhesus monkeys, indicating that TTV truly infects hepatocytes^[134]. However, TTV DNA titers in sera of TTV-infected patients decreased to undetectable levels during immunosuppression following bone marrow transplantation^[122]. Moreover, liver cells have been found to contain only TTV DNA and not mRNA^[123], suggesting that TTV replicates in hematopoietic cells rather than liver. Southern blot analysis argues against integration of the TTV genome into the genomes of human hematopoietic tumor cells, obtained from bone marrow aspirates, lymph nodes, or human hepatocellular carcinoma^[91,135]. Although, TTV DNA is frequently detected in PBMC of infected individuals^[58,120], double-stranded replicative intermediates have not been detected in PBMC.

TTV, a new member of the *Circoviridae* family, has not been cultured *in vitro* and its pathogenic potential is still not clear. A cell line for isolating and cultivating TTV will significantly accelerate the research on TTV. Human lymphoblastoid cells, particularly B cells, transformed with oncovirus such as Epstein-Barr virus, may be useful for culturing TTV. Because DNA viruses are often found integrated into host genomic DNA as has been reported for hepatitis B virus^[136-139], the possible integration of TTV DNA into the genome of hepatic cells was investigated. When a 2.2 kb TTV probe was used in a Southern blot analysis of liver genomic DNA, no signal was obtained. Thus, TTV DNA was not found to be integrated into hepatocyte chromosomes, and the liver apparently was not the site of TTV replication for this particular case. Similarly, Yamamoto *et al.*^[91] have reported the absence of viral replication in hepatocytes of TTV infected cases of hepatocellular carcinoma. These data, taken together, suggest that the site of TTV replication occurs in the bone marrow rather than in the hepatocytes, and that TTV

infection was the cause of the aplastic anemia. Similar findings suggesting TTV replication in bone marrow have been obtained by other researchers^[88].

ANTI VIRAL THERAPY

After IFN-alpha administration with a regimen of 6 MU thrice a week for 24 wk followed by 3 MU thrice a week for 12 wk, 24 of 50 (48%) concurrent TTV-infected patients achieved complete clearance of TTV DNA 6 mo after the cessation of therapy, with 7%-8% TTV spontaneous clearance rate reported annually in previous reports^[77]. Other studies indicated that IFN-alpha has a potential antiviral effect on TTV. In previous studies, IFN therapy was effective against TTV with an eradication rate of 45%-55%^[140,141]. Transient disappearance of TTV viremia during IFN-alpha therapy was observed in some reports, demonstrating the direct antiviral effects of IFN-alpha on the suppression of TTV. Nevertheless, delayed TTV clearance (TTV DNA positive at E/T and negative after cessation of therapy) that had not been reported previously occurred in few patients. Delayed complete virological response was observed in chronic hepatitis B patients after the end of therapy with Thymosin alpha 1^[142] or IFN^[143] that revealed immune modulation effects. Since TTV is a DNA virus as HBV, delayed clearance of TTV after IFN therapy may indicate that immune modulation plays an important role. The findings in the present study implied that both antiviral effects and immunomodulatory actions of IFN-alpha are important on the eradication of TTV. Further studies are needed to investigate and clarify the actual mechanism of responsiveness of TTV to IFN-alpha.

In evaluating the clinical characteristics and virological features related to clearance of TTV after IFN-alpha therapy, the viral clearance at the E/T was the only important factor associated with clearance of TTV viremia. Neither the pretreatment ALT levels nor the histopathology were predictors for TTV clearance. Besides, there was no correlation between response of HCV and TTV. All the results may indicate the difference in virologic kinetics and mechanism of IFN effects between TTV and HCV that influence the response and resistance to IFN-alpha. The ALT levels at E/T or 6 mo after cessation of treatment were not related to TTV but HCV viremia also denied the hepatopathic effects of TTV infection.

OUTLOOK ON TTV

From the compilation of published reports on this newly characterized virus and its global status, it is evident that TTV is prevalent in several countries of the world. As such, it is not involved in causation of a serious problem in the body and simply acts as a bystander without much impact of its single or co-infection with other viruses. Of course, attempts are still going on to find out exact clinical implications of TTV infection. Much is already known about the molecular biology of the virus, yet there still remains a need to develop simple techniques based on molecular and immunodiagnosics to diagnose TTV infection in all categories of laboratories. This will facilitate

studies on TTV in more detail and at several places to unravel mysteries related to this infection.

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Increased expression of angiogenin in gastric carcinoma in correlation with tumor angiogenesis and proliferation

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INTRODUCTION

Primary gastric carcinoma is one of the most common malignant tumors in China. The mortality rate is as high as 25.53/100 000, accounting for 23.93% of all the deaths caused by malignant tumors^[1]. Invasion and metastasis are the main cause for the death of cancer patients. On the other hand, invasion and metastasis of malignant tumors are closely related with angiogenesis of tumor tissue. Angiogenin (ANG) was originally isolated from serum-free supernatants of a cultured human colon adenocarcinoma cell line (HT-29) by Fett etcetera in 1985^[2]. ANG possesses a ribonuclease activity^[3]. Data have shown that in human colorectal cancer^[4], pancreatic cancer^[5], and other tumors, the expression of ANG is upregulated. However, the function of ANG in tumor angiogenesis is still unknown^[6]. We used RT-PCR and immunohistochemistry to detect the expression of ANG, CD34 and vascular endothelial growth factor (VEGF) in 68 primary gastric carcinomas and the surrounding nontumorous tissues, analyzed their correlation with each other, and explored the functional role of ANG in angiogenesis and growth of gastric carcinoma.

MATERIALS AND METHODS

Materials

The study enrolled 68 human gastric carcinoma (HGC) patients (56 men and 12 women; Ages, mean \pm SD, 60 \pm 11 years) who underwent gastric surgical resection at The First Affiliated Hospital of Fujian Medical University between March and July of 2002. The specimens for RT-PCR included both cancer tissues and the surrounding nontumorous tissues. The specimens for immunohistochemistry were taken simultaneously. All specimens were 40 g/L formaldehyde-fixed, paraffin-embedded and sliced into 4 μ m sections consecutively. None of these patients had preoperative radiative therapy or chemotherapy. All the diagnoses were confirmed by pathology reported by two pathologists. Clinical data of all the patients were reviewed. Histology type, lymph node metastasis stage and TNM stage of each case were determined according to WHO tumor classification standards (stomach cancer)^[7]. Twenty-seven patients had

Abstract

AIM: To investigate the implication of angiogenin (ANG) in the neovascularization and growth of human gastric carcinoma (HGC).

METHODS: ANG mRNA expression in HGC specimens obtained by surgical resection from patients with HGC were examined by RT-PCR. ANG, Ki-67, VEGF protein expression and microvessel density (MVD) in HGC specimens were detected by immunohistochemistry.

RESULTS: RT-PCR showed significantly higher ANG mRNA expression (0.482 ± 0.094) in HGC tissues than in the surrounding nontumorous tissues (0.276 ± 0.019 , $P = 0.03$). MVD within tumorous tissues increased significantly with ANG mRNA expression ($r = 0.380$, $P = 0.001$) and ANG protein expression ($P < 0.01$). The ANG expression levels of cancer tissues were positively correlated with VEGF ($P < 0.01$) and the proliferation index of cancer cells ($P < 0.01$).

CONCLUSION: ANG is one of the neovascularization factors of HGC. ANG may work in coordination with VEGF, and promote the proliferation of HGC cells.

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Key words: Angiogenin; Gastric carcinoma; Vascular endothelial growth factor; Angiogenesis

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well-moderately differentiated adenocarcinoma, and 41 patients had poorly-undifferentiated adenocarcinoma. Fourteen patients were node-negative; 46 had first station lymph node metastasis, and 8 had second or distant lymph node metastasis. Twelve tumors were TNM I, 25 tumors were TNM II, 20 tumors were TNM III, and 11 tumors were TNM IV.

Methods

RNA extraction: Specimens were taken within 30 min after resection, and were stored at -80°C . RNA was extracted from HGC tissue and corresponding nontumorous gastric tissue using Trizol extraction reagent kit (Gibco BRL, USA), according to the instructions of the manual. Concentration and purity of RNA were determined by spectrophotometric method. Integrity of RNA was determined by electrophoresis.

RT-PCR and analysis of the products: ANG primers were synthesized by the Bioasia Biotechnology Company using the method as described previously^[10]. Each tube contained $5 \times$ buffer $5 \mu\text{L}$, 10 mmol/L dNTP $0.5 \mu\text{L}$, DTT $1.25 \mu\text{L}$, primers (sense, antisense) $4 \mu\text{L}$, RNA template $1 \mu\text{g}$, mix-enzyme $0.5 \mu\text{L}$, DEPC solution, with a total volume of $25 \mu\text{L}$ (Titan One Tube RT-PCR system, Roche, Germany). RT-PCR reaction condition is as follows: at 58°C for reverse transcription for 30 min, 94°C incubation for 2 min to terminate reverse transcription, followed by 30 cycles at 94°C for 30 s, at 52°C for 30 s, and at 72°C for 60 s; With a final extension at 72°C for 7 min. RT-PCRs were run in a Bio-rad thermocycler. The results were analysed in a Bio-rad GelDoc-1000 system, relative to the levels of β actin as the control.

Immunohistochemistry: ANG multiclonal antibody 1:25 was purchased from American Santa Cruz Co. VEGF-C multiclonal antibody (ready to use) was provided by American Zymed Co. Monoclonal antibody of CD34 (QBEnd/10, ready to use) and monoclonal antibody of Ki-67 (MIB-1, 1:50) were supplied by American NeoMarkers Co. All operations were done according to the instructions of the manufacturers. Positive specimens were used as positive controls and PBS in substitution of the first antibody was used as a negative control at the same time.

ANG protein expression^[8]: Cytoplasm of gastric carcinoma cells was stained brown. According to the staining intensity of the tumor cells, the staining was graded as (-), when staining was weaker than negative control; (+), for light staining; (++) , for moderate staining; and (+++) , for strong staining.

MVD of HGC tissues^[9]: Gastric carcinoma vascular endothelial cells were stained brown. The isolated brown and yellow blood vessel endothelial cells or cell clusters in gastric carcinoma tissues were regarded as a single microvessel (Figure 1A). Areas with the highest microvessel densities were selected under $10 \times$ microscopic magnification. Then the numbers of microvessels stained by CD34 antibody in 10 vision fields were counted under $400 \times$ magnification (0.916 mm^2 per visual field), respectively. The average of 3 highest values was taken as MVD. Indistinguishable or indistinct cells were excluded.

VEGF protein expression: Membrane and cytoplasm

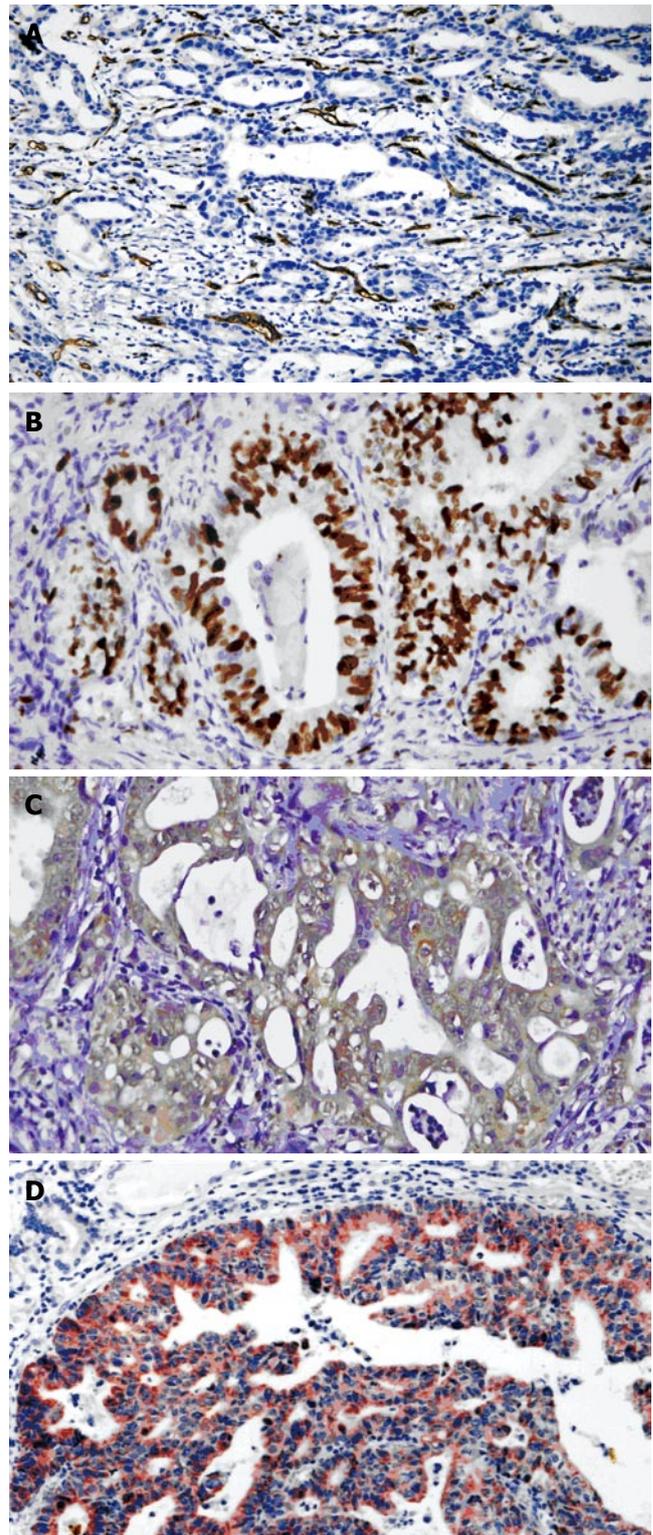


Figure 1 Expression of CD34, Ki-67, ANG and VEGF in gastric carcinoma tissue. A: CD34 (S-P $\times 200$); B: Ki-67 (S-P $\times 400$); C: ANG (EnVision $\times 400$); D: VEGF (S-P $\times 200$).

of gastric carcinoma cells were stained rose. The staining intensity of the tumor cells was classified as: light staining (+), moderate staining (++) , deep staining (+++).

Proliferation index of tumor cells: The nuclei of the positive cells were stained deep brown (Figure 1B). The number of positive cells among 1000 tumor cells was counted per slide and taken as the tumor cell proliferation index.

Table 1 ANG and ANG mRNA expression and clinicopathological characteristics of gastric carcinoma

Characteristics	n	ANG mRNA (mean ± SE)	ANG			
			+	++	+++	++++
Age (yr)						
< 60	37	1.727 ± 0.287	3	7	19	8
≥ 60	31	2.634 ± 0.698	0	4	16	11
Site						
Cardia	25	2.866 ± 0.610	0	1	14	10
Corpus	10	1.310 ± 0.166	1	1	4	4
Antrum	25	2.366 ± 0.737	2	6	13	4
Others	8	1.704 ± 0.508	0	3	4	1
Differentiation						
Well-moderate	27	1.986 ± 0.247	1	2	15	9
Poor	41	2.242 ± 0.570	2	9	20	10
Depth of invasion						
Lamina and muscularis propria	12	1.384 ± 0.148	2	2	6	2
Visceral peritoneum	56	2.302 ± 0.429	1	9	29	17
Lymph node metastasis						
(-)	14	1.722 ± 0.186	1	1	9	3
(+)	54	2.245 ± 0.445	2	10	26	16
TNM stage						
I	12	1.756 ± 0.212	1	1	8	2
II	25	1.621 ± 0.273	2	3	10	10
III-IV	31	2.708 ± 0.739	0	7	17	7

Statistical analysis

Statistics software package was used for analysis. *t* test, analysis of variance, chi-square test and correlation analysis were used. $P < 0.05$ was taken as significance.

RESULTS

ANG mRNA expression in HGC tissues

ANG mRNA and its inner control β -actin were detected both in gastric carcinoma tissues and in corresponding nontumorous gastric tissues of all 68 cases. The RT-PCR products of ANG and β -actin were seen at 402 bp and 234 bp by gel electrophoresis (Figure 2). The expression of ANG mRNA was higher in 68 gastric carcinoma tissues (0.4822 ± 0.0943) than in corresponding nontumorous gastric tissues (0.2758 ± 0.0187 , $P < 0.05$). Although ANG mRNA expression was higher in poorly differentiated carcinomas, in carcinomas invading visceral peritoneum, carcinomas with lymph node metastasis and TNM stage III-IV than in well-moderately differentiated adenocarcinoma, in carcinomas invading lamina and muscularis propria, carcinomas without lymph node metastasis and TNM stage I-II, no statistical difference was found (Table 1).

ANG expression in HGC tissues

Immunohistochemistry staining showed that ANG located at the cytoplasm of gastric carcinoma cells as brown particles (Figure 1C). Over 90% of the gastric carcinoma cells were stained positive. The ANG expression level had no correlation with the age of patients, location, histological type, lymph node metastasis, and clinical stage of the tumor (Table 1).

ANG expression and the MVD of tumor tissues

ANG mRNA expression ratio of HGC tissues and the corresponding nontumorous gastric tissues (T/N) was

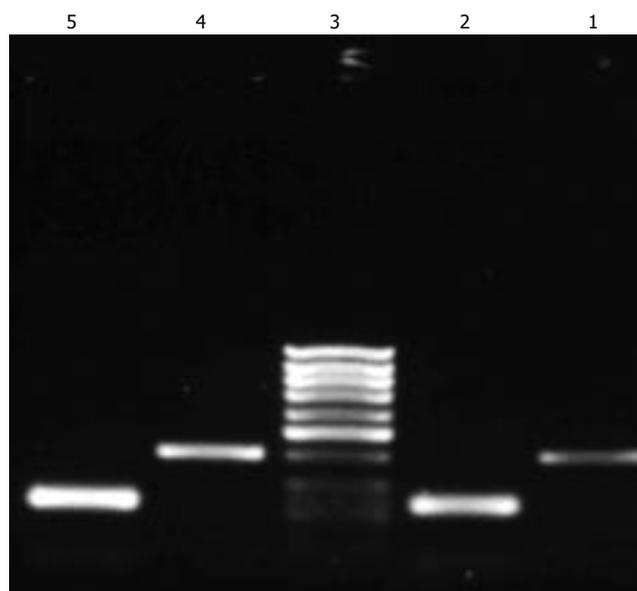


Figure 2 RT-PCR product electrophoresis. 1: β -actin of gastric carcinoma tissue, 234 bp; 2: ANG of gastric carcinoma tissue, 402 bp; 3: molecular weight marker; 4: β -actin of paired adjacent gastric mucosas, 234 bp; 5: ANG of paired adjacent gastric mucosas, 402 bp.

significantly correlated with the MVD of gastric carcinoma tissue. With the increase of ANG mRNA expression, MVD elevated also. A significant positive correlation was found between them ($r = 0.380$, $P < 0.01$). Also, with increase of ANG expression of gastric carcinoma tissues, MVD rose as well. The MVD of the ANG strong staining group (308.37 ± 25.57) was significantly higher than that of the negative group (196.00 ± 31.34) ($P < 0.01$).

Correlation between VEGF and ANG expression in HGC

The ANG expression in gastric carcinoma tissues was positively correlated with the expression of VEGF (Figure 1D) (Pearson correlation coefficient = 0.3490, $P < 0.01$).

Correlation between tumor proliferation index and ANG expression in HGC

The ANG expression levels of cancer tissues were positively correlated with the proliferation index of cancer cells ($P < 0.01$). The proliferation index of cancer cells of the group with strong ANG expression (579.58 ± 31.38) was much higher than that of the group with weak ANG expression (341.33 ± 84.01).

DISCUSSION

The up-regulation of expression of angiogenic factors in tumor tissue plays the role of switch-on in neoplastic angiogenesis. Research showed that hypoxia could induce up-regulation of ANG expression in human malignant melanoma, whereas it could induce down-regulation of ANG expression in normal melanocytes^[10]. It has been shown that ANG mRNA expression is significantly higher in human colorectal cancer^[4], pancreatic cancer^[5], malignant melanoma^[10], bladder carcinoma^[11] and other tumors than in normal tissues. Our results are similar to these. The ANG expression levels of gastric

carcinoma tissues were significantly higher than that in the surrounding nontumorous gastric mucosae. Both ANG mRNA and protein expression levels had no significant correlation with the patients' age, location, histological type, lymph node metastasis, and clinical stage of the tumor^[12]. Studies on pancreatic cancer^[5], invasive breast carcinoma^[13] and invasive cervical cancer^[14] have obtained similar results. In malignant melanoma, ANG expression is upregulated around necrotic areas and invasive borders of tumors, while necrotic tissues do not express ANG. This suggests that hypoxia is the reason for the upregulation of ANG in gastric carcinoma tissues.

Tumor angiogenesis is the precondition of tumor development. Tumor MVD is recognized as an important index for judging tumor angiogenesis, which is a multi-step process. Under the effect of angiogenic factors, endothelial cells proliferate and migrate to form new vessels, and new blood vessel networks. This study showed a positive correlation between ANG expression levels and tumor vascularity evaluated by MVD, suggesting ANG contributes to the angiogenesis of HGC. In addition, previous studies have shown ANG contributes to the neovascularization of hepatocellular carcinoma^[15], human colorectal cancer, and malignant melanoma^[10]. ANG is one of the efficient angiogenic factors, and the only angiogenic factor with RNase activity^[16]. There are two kinds of ANG-binding proteins on endothelial cells. One is actin, the other is a 170-kDa ANG-binding protein. Binding of ANG to cell surface actin causes activation of a cell-associated protease system and cell invasion. After the cells are activated, they migrate and invade the basement membrane, resulting in a decrease in the local cell density in the vicinity of the migrating cells, which may trigger the putative 170-kDa receptor gene expression in the remaining cells. These cells become ANG-responsive and divide to fill the space left by the migrating cells. The expression of the receptor gene is turned off when the gap is filled and cells become more dense. It may well be possible that the ANG-induced formation of a new capillary network is largely regulated by such cell density-dependent expression of the ANG receptor gene^[3].

ANG is required for cell proliferation induced by various other angiogenic proteins including acidic and basic fibroblast growth factors (aFGF and bFGF), epidermal growth factor (EGF), and vascular endothelial growth factor (VEGF). Angiogenin-stimulated rRNA transcription in endothelial cells may thus serve as a crossroad in the process of angiogenesis induced by various angiogenic factors^[17].

We found a positive correlation between ANG protein expression and VEGF expression in HGC tissues, suggesting ANG and VEGF may work in coordination in HGC angiogenesis. Asthmatic children have significantly higher levels of VEGF and angiogenin than healthy control children. A significant positive correlation exists between both angiogenic factors^[18]. Moreover, a correlation has been found between VEGF and ANG levels during fetal development and in maternal diabetes^[19]. Gonadotropin can not only stimulate the secretion of ANG by granulosa cells, but can also stimulate the secretion of VEGF by ovarian follicles. ANG and VEGF may also

work together in the angiogenesis of corpus luteum^[20]. Meanwhile, it was also found that hypoxia could induce the expression of VEGF^[16]. Hypoxic induced factor-1 (HIF-1) binds VEGF oxygen sensitive enhancer under hypoxic conditions. Through the activation of c-Src, it promotes the transcription and expression of VEGF. Expression of ANG may be up-regulated in response to hypoxia within the tumor. A significant increase in the secretion and mRNA expression of ANG from both term placental explants and trophoblast cultures subjected to hypoxia *in vitro* was observed^[21]. One study showed that ANG expression in invasive carcinomas is significantly positively correlated with HIF-1 alpha and the HIF-1 alpha target gene DEC-1^[22]. Hypoxia can also induce upregulated expression of ANG in corpus luteum granulosa cells. This is related to two HIF binding sites on the ANG promoter^[20]. Moreover, under hypoxic conditions, the ANG and VEGF secreted by renal proximal tubular epithelial cells may modulate angiogenesis and vascular remodeling in the renal *interstitium* via an increase in the production of HIF-1^[23]. Accordingly, hypoxia in HGC tissues may stimulate ANG and VEGF expression through the HIF-1 pathway. ANG and VEGF may work coordinately in the angiogenesis of HGC.

We found that ANG expression levels were closely correlated with rapid growth and metastasis of malignant tumors. Pancreatic cancers with high serum ANG (sANG) levels had larger volumes than those to low sANG levels^[5]. ANG expression in the gastric carcinoma tissues was significantly and positively correlated with the proliferation index of cancer cells. This suggests ANG could promote the growth of tumor cells. Studies have found that ANG can specifically combine with the leukemia cell line TF-1 and dose dependently promotes the growth of the leukemia cells^[24]. ANG is constitutively translocated into the nucleus of HeLa cells where it stimulates rRNA transcription, ribosome biogenesis, proliferation and tumorigenesis^[25].

In summary, ANG contributes to gastric carcinoma angiogenesis, and promotes the growth of tumor cells. Inhibition or blockage of the expression of ANG does not only reduce tumor angiogenesis, but also inhibits the growth of tumors directly. These have potential implications in tumor treatment.

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COLORECTAL CANCER

Prolonged exposure of colon cancer cells to the epidermal growth factor receptor inhibitor gefitinib (Iressa™) and to the antiangiogenic agent ZD6474: Cytotoxic and biomolecular effects

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Abstract

AIM: To analyze the biological effects of prolonged *in vitro* exposure of HT-29 and LoVo colon cancer cell lines to gefitinib (Iressa™), an inhibitor of epidermal growth factor receptor (EGFR) activity, and ZD6474, an inhibitor of both KDR and EGFR activities.

METHODS: Cells were treated with each drug for up to 2 wk using either a continuous or an intermittent (4 d of drug exposure followed by 3 d of washout each week) schedule.

RESULTS: In both cell types, prolonged exposure (up to 14 d) to gefitinib or ZD6474 produced a similar inhibition of cell growth that was persistent and independent of the treatment schedule. The effects on cell growth were associated with a pronounced inhibition of p-EGFR and/or p-KDR expression. Treatment with gefitinib or ZD6474 also inhibited the expression of EGFR downstream signal molecules, p-Erk1/2 and p-Akt, although the magnitude of these effects varied between treatments and cell lines. Furthermore, expression of the drug resistance-related protein ABCG2 was shown to significantly increase after 14 d of continuous exposure to the two drugs.

CONCLUSION: We conclude that long-term exposure of colon cancer cells to gefitinib and ZD6474 does not modify their cytotoxic effects but it might have an effect on sensitivity to classical cytotoxic drugs.

INTRODUCTION

Recently, a greater understanding of the molecular basis of cancer has fostered the development of rationally designed molecular-targeted therapies with agents that have been shown to prevent cell proliferation, differentiation and survival through the inhibition of receptor tyrosine kinases (TKs), such as epidermal growth factor receptor-1 (EGFR) and vascular endothelial growth factor receptors (VEGFRs)^[1,2].

Drugs that specifically target these receptors act *via* inactivation of the tyrosine kinase function of EGFR receptors resulting in lack of recruitment and phosphorylation of several intracellular substrates. A major downstream signalling route involved in this process is the Ras-Raf-MAPK pathway finally leading to ERK1 and 2 inactivation^[3,4]. Another important target in EGFR signalling is PI3K and the downstream AKT protein transducing signals to the cascade of survival and motility^[5,6]. Recently, the relevant role of the oncosuppressor gene PTEN in uncoupling some of these signalling pathways and thus generating gefitinib resistance has also been stressed^[7].

Gefitinib (Iressa) is a well-known oral EGFR inhibitor that is able to reduce tumour growth and the formation of metastases in a range of human cancer cell lines and human tumour xenografts^[8,9]. In the clinical setting, gefitinib monotherapy has demonstrated antitumour activity in patients with recurrent or refractory non-small-cell lung cancer^[10,11] and it has been approved for clinical cancer treatment in several countries. ZD6474 is a novel, orally available antiangiogenic agent that selectively targets two

key tumour growth pathways by inhibiting VEGFR and EGFR tyrosine kinase activities. Preclinical studies have shown ZD6474 to be a potent inhibitor of VEGF-induced endothelial cell proliferation, tumour-induced angiogenesis and tumour growth^[12].

Combining gefitinib or ZD6474 with other biological or cytotoxic agents has resulted in enhanced antitumour effects *in vitro* and *in vivo*^[13-17]. Several ongoing clinical trials are therefore investigating the clinical efficacy of these targeting molecules when administered (1-21 or 1-28 d) in combination with agents such as taxols, gemcitabine, cisplatin, oxaliplatin, 5-FU and irinotecan [www.clinical-trials.gov]. However, we have recently demonstrated that the effect of gefitinib used in combination with some cytotoxic drugs, can be schedule-dependent and have concluded that only extensive analysis of their main pathways of action could help in the design of an optimal multi-drug therapy^[13,15,16]. The great influence that schedules can have in combination treatments with TK-inhibitors and other cytotoxic drugs is also evinced by the fact that EGFR inhibitors can variously interact with proteins involved in resistance to some conventional cytotoxic drugs^[18,19]. In particular, ABCG2 and mdr-P glycoprotein (P-gp), belonging to ATP-binding cassette (ABC) transporters, are involved in sensitivity to topoisomerase I inhibitors^[20] and anthracyclines^[21].

In vitro data analyzing the activity of TK inhibitors on cell growth, apoptosis induction or cell cycle and target modulation are mainly limited to very short term cell exposures to drugs, generally lasting no longer than 5 d^[22-24]. Although such exposure times are useful to clarify the molecular mechanisms of action of this class of drugs, they do not account for some major cell mechanisms controlling the expression and function of EGFR receptors^[25]. Some such mechanisms that are known to produce EGFR down-regulation are endocytosis, pH-sensitive dissociation, dephosphorylation by PTP1B, trafficking to the lysosome, *etc.*^[26-29].

Data from *in vivo* studies on the effects of prolonged exposure to TK inhibitors are scant. An *in vivo* study on long-term exposure confirmed that TK inhibitors are able to reduce tumour growth when utilized alone or in association with other drugs^[9,30] but at least one other study has demonstrated that tumour cell lines can develop resistance to gefitinib^[31]. The only *in vitro* report analyzing the pharmacological and biological effects of prolonged exposure to an EGFR inhibitor supports the hypothesis that duration of cell exposure to such a drug is important in modulating its antitumour effect and synergism with other drugs^[32].

The aim of this study was to investigate the cytotoxic and biomolecular effects of different gefitinib and ZD6474 long-term exposure modalities on colon cancer cell lines and, whether phosphorylation of their specific targets (EGFR and/or KDR), activity of downstream signalling molecules and multidrug resistance proteins were modified in an exposure time-dependent manner.

MATERIALS AND METHODS

Drugs and chemicals

Gefitinib and ZD6474 were provided by AstraZeneca Phar-

maceuticals (Macclesfield, UK). Stock solutions were prepared at 20 $\mu\text{mol/L}$ in dimethyl sulphoxide (DMSO) and stored in aliquots at -20°C . Further dilutions were made in F-12/HAM or McCoy's medium supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, 50 000 U/L penicillin, and 80 $\mu\text{mol/L}$ streptomycin.

Cell lines

Two colon cancer cell lines of human origin were used, LoVo and HT-29. Cells were routinely cultured in F-12/HAM (LoVo) or McCoy's (HT-29) medium, supplemented as above, in a humidified incubator at 37°C with a 50 mL/L CO_2 atmosphere. Cells were trypsinized once a week with trypsin 0.25% ethylenediaminetetraacetic acid 0.02%, and the medium was changed twice a week. Doubling times were 20 ± 1 h for HT-29 cells and 24 ± 1 h for LoVo cells.

HPLC drug analysis

Gefitinib or ZD6474 (1-100 $\mu\text{mol/L}$) were incubated in F-12/HAM or McCoy's medium, supplemented as above, in a humidified incubator at 37°C with a 50 mL/L CO_2 atmosphere for 1, 5 and 7 d, after which HPLC analysis was performed. The stock solutions of each drug in DMSO were used as controls. The HPLC consisted of an LC9010 system coupled with a UV detector (Varian Inc, Palo Alto, CA, USA). Purified drug was eluted from an Aspec Bond Elut-C2 column (Varian Inc) using a solution of triethylamine and acetonitrile (76:24 [v/v]) adjusted to pH 3.0 with 0.2 mol/L phosphoric acid.

Evaluation of cytotoxicity

Determination of the IC_{50} of gefitinib or ZD6474 was performed using the 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazoliumbromide (MTT) assay. On d 1, 10 000 cells/well in a volume of 200 μL were added to 96-well plates. In each plate, one column contained cells not exposed to the drug (control), and five columns contained cells exposed to increasing concentrations of the drug. Each drug concentration was repeated in six identical wells. On d 2, gefitinib or ZD6474 (0.01, 0.1, 1, 10 and 100 $\mu\text{mol/L}$) was added allowing for different times of drug exposure (18 h to 3 d). Results were expressed as dose-effect curves with a plot of the fraction of unaffected (surviving) cells *versus* drug concentration. The IC_{50} was defined as the drug concentration yielding a fraction = 0.5 of affected (not surviving) cells, compared with untreated controls.

Cell growth inhibition following prolonged drug exposure

LoVo and HT-29 cells were exposed to gefitinib (0.12 $\mu\text{mol/L}$ and 1.2 $\mu\text{mol/L}$, respectively) and ZD6474 (0.6 $\mu\text{mol/L}$ and 5 $\mu\text{mol/L}$, respectively). These sub-toxic concentrations induced 30% cell growth inhibition after 3 d of continuous drug exposure. Cell survival was determined by cell counts after 7 and 14 d of drug exposure, using either a continuous or intermittent (4 d with drug followed by a 3-d washout) schedule. Controls were untreated cells which, at a quite complete confluence, were counted, divided, and plated again at 70% of confluence. Results were expressed as percentage of cell survival compared with untreated controls.

Table 1 AUC and retention time of gefitinib or ZD6474 following incubation for 7 d at 37°C, by HPLC analysis

Drug	Medium	Concentration (μmol/L)	Retention time (min)	AUC
Gefitinib	DMSO	100	2.47 ± 0.03	1.30 ± 0.20
	Complete medium	100	2.52 ± 0.05	1.27 ± 0.16
ZD6474	DMSO	100	1.75 ± 0.02	0.88 ± 0.08
	Complete medium	100	1.71 ± 0.04	0.86 ± 0.10

Table 2 IC₅₀ of gefitinib and ZD6474 in HT-29 and LoVo cells at various times of incubation

Exposure time	Gefitinib (μmol/L)		ZD6474 (μmol/L)	
	LoVo cells	HT-29 cells	LoVo cells	HT-29 cells
18 h	48.5 ± 2.5	> 100	16 ± 5.1	80 ± 4.8
1 d	29 ± 3.1	> 100	13 ± 2.6	59 ± 3.6
2 d	16.5 ± 1.5	> 100	8.2 ± 3.8	19 ± 1.8
3 d	7.3 ± 0.9	23.6 ± 4.1	3.5 ± 0.9	10 ± 0.4

Each experiment was conducted in triplicate.

Cell-cycle perturbation and apoptosis

LoVo and HT-29 cells were exposed to gefitinib (0.12 μmol/L and 1.2 μmol/L, respectively) and ZD6474 (0.6 μmol/L and 5 μmol/L, respectively) for 7 and 14 d, using either a continuous or intermittent (4 d with drug followed by a 3-d washout) schedule. Cells were then harvested, washed twice in ice-cold PBS (pH 7.4), fixed in 4.5 mL of 70% ethanol at -20°C and washed once again in ice-cold PBS. After resuspension of the pellet in PBS containing 1 mg/mL RNase and 0.01% NP-40, cellular DNA was stained with 50 μg/mL propidium iodide (Sigma-Aldrich, St Louis, MO, USA). Cells were stored in ice for 60 minutes before analysis. Cell cycle and apoptosis determinations were performed using a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA), and data were interpreted using the ModFit software provided by the manufacturer.

Immunoprecipitation and Western blot analysis of targets after prolonged drug exposure

Expressions of EGFR, KDR, Akt, p-Akt, Erk1/2, p-Erk1/2, PTEN, ABCG2 and PgP were determined by Western blot analysis using β-actin as the standard protein. Expressions of p-EGFR and p-KDR were determined by immunoprecipitation and Western blot. Protein bands were quantified by densitometric analysis followed by analysis with Quantity One software (BioRad, Hercules, CA, USA). The primary antibodies used were anti-EGFR (clone 13) and anti-KDR (clone A-3) from BD Transduction Laboratories (San Diego, CA, USA); Anti-Akt, anti-p-Akt, anti-Erk1/2 and anti-p-Erk1/2 (clone E10) from Cell Signaling Technology (Beverly, MA, USA); Anti-ABCG2 (clone BXP-21) from Alexis Corporation (Lausen, Switzerland); Anti-PgP (clone F4) and anti-β-actin from Sigma-Aldrich; And anti-phosphotyrosine polyclonal antibody PY99 and anti-PTEN (A2B1) from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The secondary antibodies used were horseradish-peroxidase-conjugated anti-mouse and anti-rabbit from Amersham Pharmacia Biotech (Uppsala, Sweden).

Statistical analysis

All the *in vitro* experiments were performed in triplicate, and results are expressed as the mean ± SD unless otherwise indicated.

RESULTS

Time-dependent drug stability

To verify gefitinib and ZD6474 stability in our

Table 3 Cell cycle and apoptosis modulation after prolonged exposure to gefitinib or ZD6474 in HT-29 and LoVo cells

Drug exposure	LoVo cells		HT-29 cells		
	Apoptosis (%)	G0/G1 (%)	Apoptosis (%)	G0/G1 (%)	
Control	0	74.3 ± 1.3	0	69.5 ± 1.4	
Gefitinib	1 wk continuous	10.1 ± 0.5	80.4 ± 1.5	7 ± 0.4	70.7 ± 1.7
	2 wk continuous	23.2 ± 0.9	87.9 ± 1.3	20 ± 1.2	77.2 ± 2.0
	1 wk intermittent	13.8 ± 0.6	75.4 ± 1.4	6.5 ± 0.3	67.1 ± 1.4
	2 wk intermittent	29.7 ± 0.8	78.0 ± 2.1	18.3 ± 1.8	69.7 ± 1.9
Control	0	74.3 ± 2.1	0	69.5 ± 1.8	
ZD6474	1 wk continuous	15.4 ± 0.6	77.6 ± 1.9	7 ± 0.9	71.7 ± 2.1
	2 wk continuous	30.3 ± 0.9	81.8 ± 2.3	15 ± 0.8	75.2 ± 2.4
	1 wk intermittent	18.3 ± 1.3	74.6 ± 2.1	7.3 ± 1.1	70.2 ± 1.8
	2 wk intermittent	29.6 ± 1.5	76.5 ± 1.5	16.4 ± 2.2	72.0 ± 1.7

Each experiment was conducted in triplicate.

experimental conditions, each drug was incubated in complete medium for 1, 5 and 7 d. The HPLC retention time and the area under the curve (AUC) for the peak of each drug were measured and compared to those of the same drugs in DMSO (standard). The HPLC retention times for the internal standards were 2.47 min for gefitinib and 1.75 min for ZD6474. Each drug showed the same retention time as its standard, irrespective of concentration, exposure time and the absence or presence of complete medium; the AUC of each peak was proportional to the drug concentration. These results confirmed the stability of gefitinib and ZD6474 in our experimental conditions. Table 1 shows the AUC and retention times of gefitinib and ZD6474 peaks obtained by HPLC after 7 d of drug exposure.

Drug-dependent cell growth inhibition and apoptosis

The IC₅₀ values for gefitinib and ZD6474 in HT-29 and LoVo cells are shown in Table 2. A time-dependent reduction in IC₅₀ was observed for both drugs, with the IC₅₀ after 3 d of exposure being 4-8 times lower than after 18 h of exposure.

Treatment with gefitinib for 1 wk, according to a continuous or intermittent schedule, did not significantly affect LoVo cell growth, and 2 wk of treatment was necessary to show an inhibitory effect (Figure 1A). By contrast, HT-29 cell growth was inhibited by gefitinib at 1 wk and to an even greater extent at 2 wk. ZD6474 induced a progressive, exposure-dependent inhibition of cell growth in both cell lines, but HT-29 cells appeared to be more sensitive than LoVo cells (Figure 1B).

As expected, prolonged exposure of the cells to gefit-

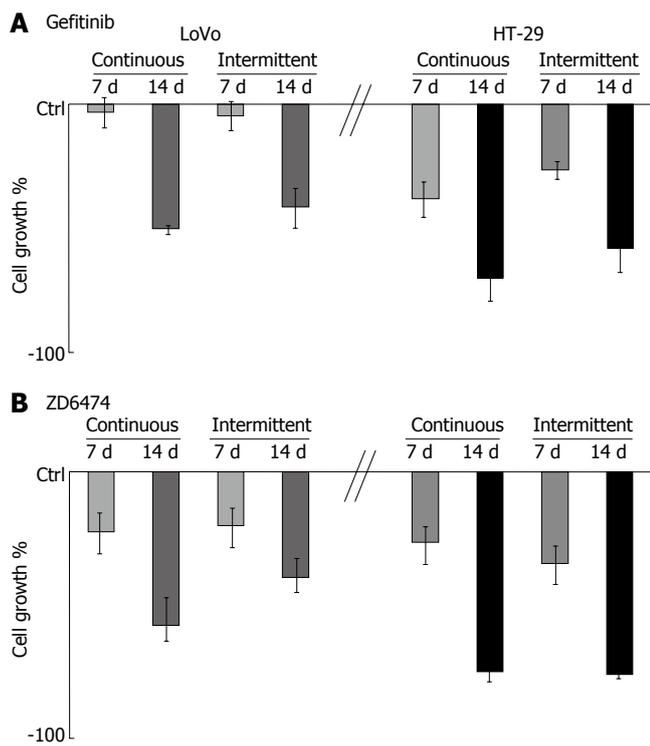


Figure 1 Drug-dependent cell growth inhibition. LoVo and HT-29 cells were incubated with gefitinib (0.12 and 1.2 $\mu\text{mol/L}$, respectively) or ZD6474 (0.6 and 5 $\mu\text{mol/L}$, respectively) for 7 and 14 d with continuous and intermittent exposure. Cell survival was determined by cell counts.

inib or ZD6474 produced an exposure-dependent increase in apoptosis (Table 3). This effect was observed with both the continuous and the intermittent treatment schedules. There was also a moderate accumulation of cells in the G0/G1 phase and this effect became more evident at 2 wk with continuous exposure (Table 3).

Modulation of TK receptor signal transduction pathways

The ability of gefitinib and ZD6474 to modulate their specific targets and downstream effectors was analyzed by measuring the expression levels of total and phosphorylated proteins.

TK receptor modulation: Prolonged exposure to gefitinib and ZD6474 of both cell lines, using either a continuous or intermittent treatment schedule, did not change the total amount of EGFR or KDR protein. HT-29 cells exposed to gefitinib for 7 and 14 d showed almost no detectable p-EGFR (Figure 2). In LoVo cells, gefitinib produced partial inhibition of p-EGFR that was appreciable only after 14 d of treatment. In both cell lines, ZD6474 almost completely inhibited p-KDR (approximately 98% inhibition compared with the control; Figure 2). ZD6474 also inhibited p-EGFR, with a greater effect in HT-29 cells than in LoVo cells.

EGFR signal transduction pathway modulation: Prolonged exposure to gefitinib and ZD6474 did not change the expression of Akt and Erk1/2 in HT-29 or LoVo cells. Gefitinib produced only modest effects on p-Akt but markedly decreased p-Erk1/2, the downstream effector of the proliferation pathway (Figure 3). Compared with the 7 d exposure, the magnitude of gefitinib-mediated

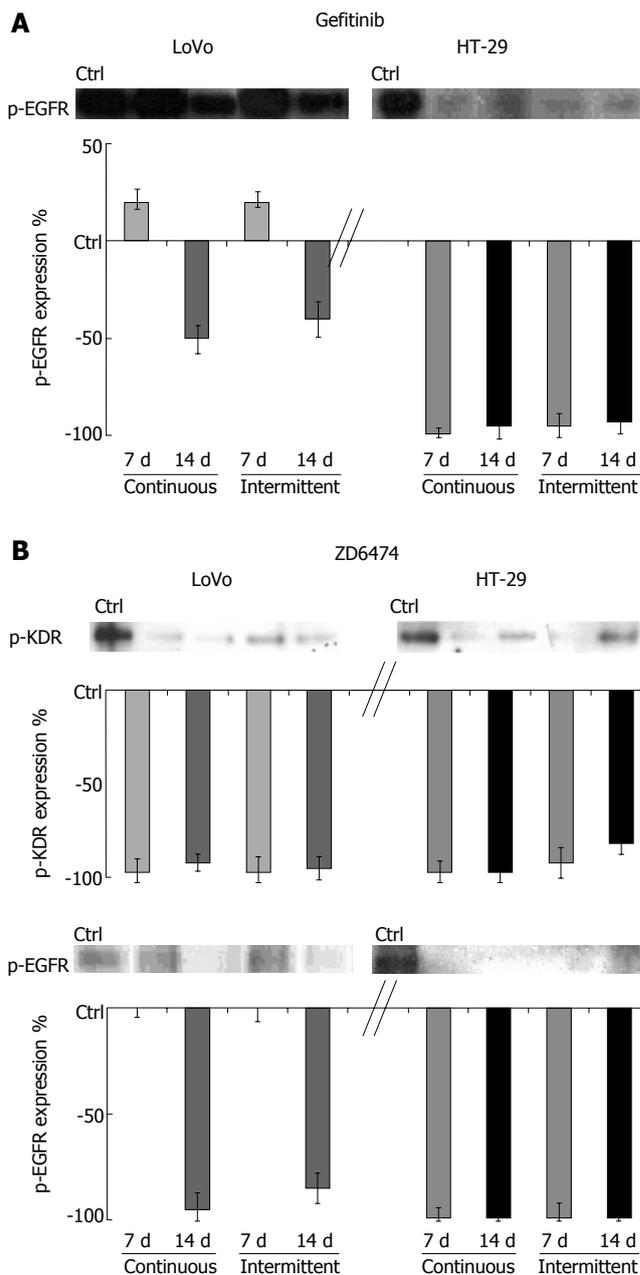


Figure 2 p-EGFR and p-KDR modulation after prolonged exposure to gefitinib or ZD6474. Cells were incubated with gefitinib or ZD6474 for 7 and 14 d with continuous and intermittent exposure. Drug-dependent modulation of p-EGFR (180 kDa) and p-KDR (195 kDa) was determined by immunoprecipitation followed by Western blotting. All data are shown relative to the baseline level (control = 0), which was similar after 7 and 14 d.

inhibition of p-Erk1/2 at 14 d was similar in the HT-29 cells, but had decreased in the LoVo cells.

Continuous or intermittent treatment with ZD6474 was associated with only a slight inhibition of p-Akt (Figure 3). ZD6474 induced a progressive and almost complete inhibition of p-Erk1/2 in HT-29 cells, but not in LoVo cells.

PTEN modulation: With a progressive increase in exposure time from 1 to 14 d, neither gefitinib nor ZD6474 modulated the total amount of PTEN in either cell line (data not shown).

Drug resistance induction

Preliminary analysis of the baseline expression levels of

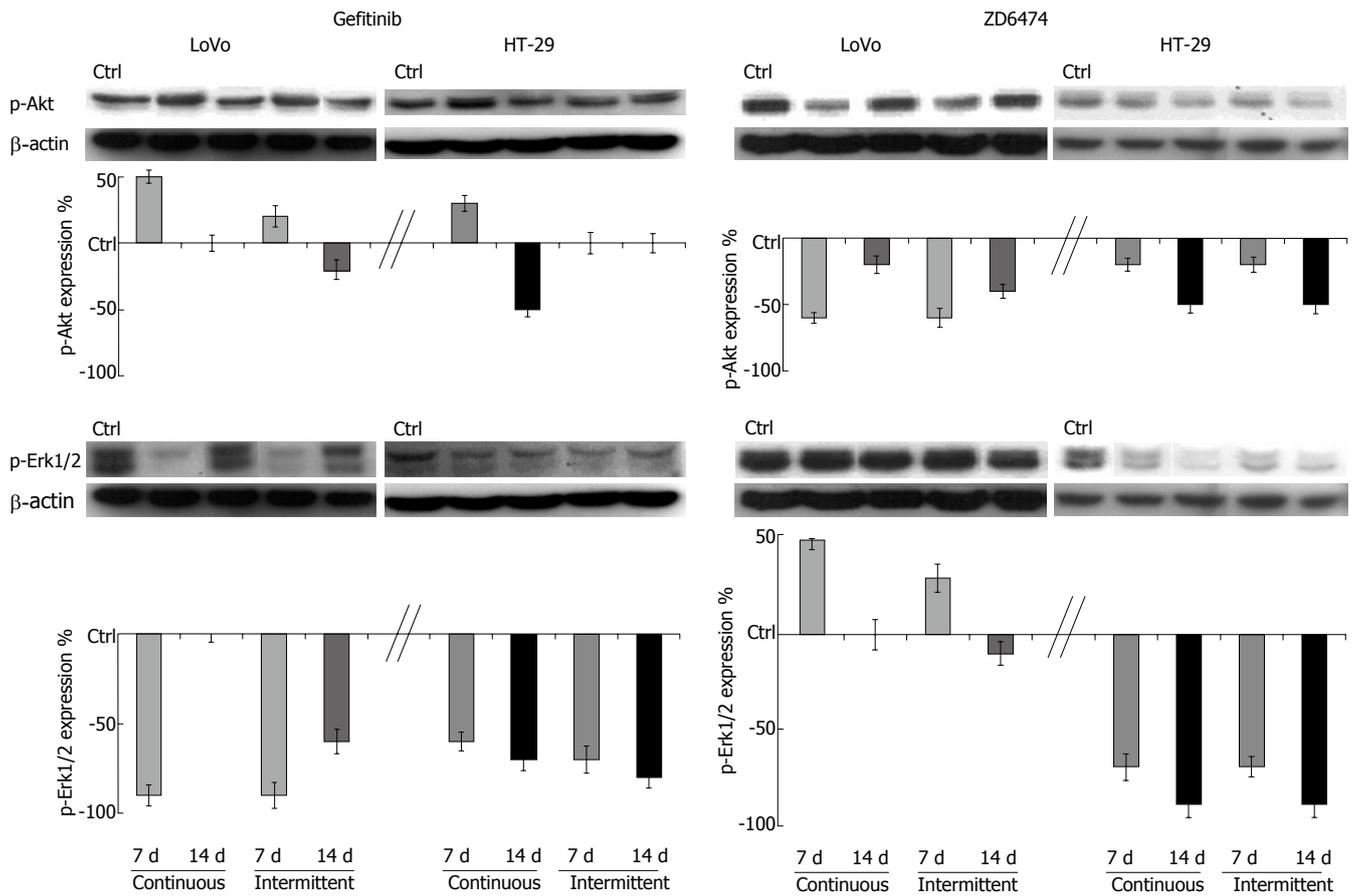


Figure 3 p-Akt and p-Erk1/2 modulation after prolonged exposure to gefitinib or ZD6474. Cells were incubated with gefitinib or ZD6474 for 7 and 14 d with continuous and intermittent exposure. Drug-dependent p-Akt and p-Erk1/2 modulation was determined by Western blotting. All data are shown relative to the baseline level (control = 0), which was similar after 7 and 14 d.

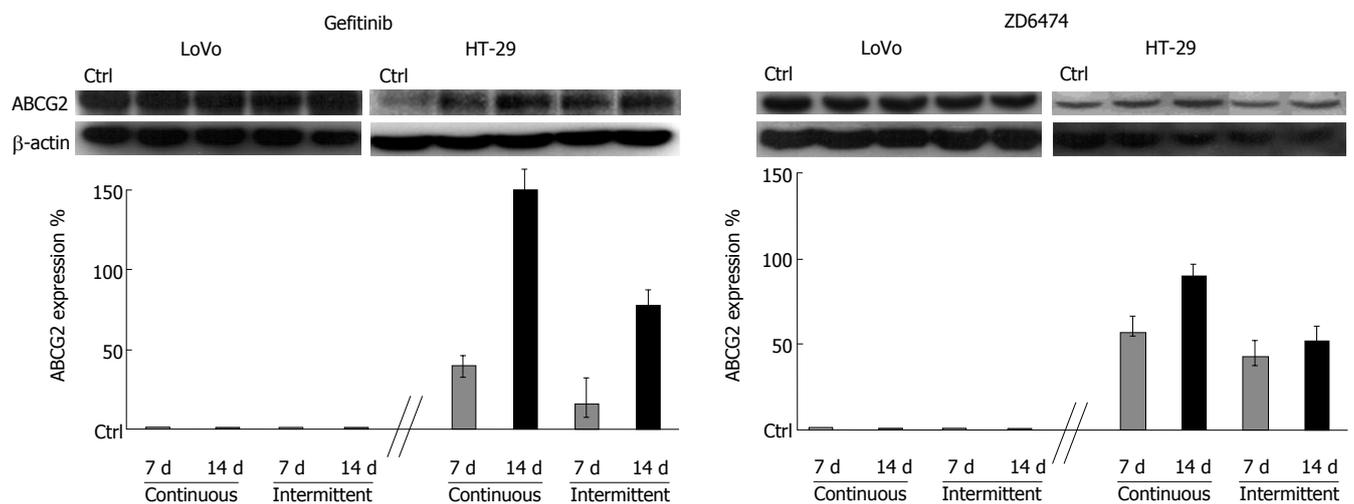


Figure 4 Increased ABCG2 expression after prolonged exposure to gefitinib or ZD6474. Cells were incubated with gefitinib or ZD6474 for 7 and 14 d with continuous and intermittent exposure. Drug-dependent increases in ABCG2 expression were determined by Western blotting. All data are shown relative to the baseline level (control = 0), which was similar after 7 and 14 d.

proteins related to drug resistance showed that ABCG2 was detectable in HT-29 and LoVo cells while P-gP was undetectable. LoVo cells also showed a higher level of ABCG2 than HT-29 cells. Interestingly, both gefitinib and ZD6474 exhibited time exposure-dependent increase in ABCG2 expression only in HT-29 cells; this effect became

evident after 5 d of drug exposure (data not shown) and was progressive until 14 d (Figure 4). Stimulation of ABCG2 expression was more evident after continuous exposure to gefitinib or ZD6474 (150% and 90% increase in ABCG2 expression, respectively) than after intermittent exposure (80% and 50% increase compared with controls, respectively).

DISCUSSION

Analysis of the biomolecular effects that the TK inhibitors, gefitinib and ZD6474, can have on tumour cells after prolonged drug exposure is instrumental in leading to optimization of their use in monotherapies and in combination with other biological or conventional cytotoxic drugs^[10,11,33,34]. Several cell effects have been demonstrated to depend directly on the modality and duration of cell exposure to these drugs; these effects include receptor expression, function of signal transduction, modulation of drug resistance proteins, etc. Nonetheless, and in spite of the clinical modalities of administration of these drugs, *in vitro* studies describing the inhibitory effect of gefitinib or ZD6474 on cell growth have considered only short drug exposures of 1-5 d^[22-24].

In our study, we assessed the effects of prolonged exposure to the TK inhibitors, gefitinib and ZD6474, on cell viability and on their specific molecular targets by directly monitoring the modulation of the phosphorylated form of EGFR and of the two effectors, Akt and Erk1/2, that are important in the cell survival and proliferation pathways, respectively.

Two weeks exposure to gefitinib resulted in up to 70% cell growth inhibition and no apparent differences between the continuous and the intermittent treatment schedules. Moreover, as already reported for erbitux^[32], gefitinib inhibited the phosphorylated forms of the receptor p-EGFR and of the downstream effector p-Erk1/2 involved in the proliferation pathway. The effects of gefitinib on p-Akt were less dramatic and appeared to be cell line-specific.

Comparison of these results with those obtained after short-term drug exposure^[13] highlighted differences in the molecular effects produced by different drug schedules. Although a high rate of cells died after 2 wk of drug exposure in our investigation, cells tried to escape the attack by an exogenous agent by further modulating the survival and proliferation pathways. These findings suggest that a combination of Gefitinib and other PI3K/Akt pathway inhibitors, such as mTor inhibitor, may produce a more powerful synergistic effect.

ZD6474 proved to be able to inhibit the growth of our two colon cancer cell lines, thus confirming similar effects previously observed in GEO cells^[23]. In both cell lines, the ZD6474-mediated inhibition of growth was associated with almost complete inhibition of p-KDR and p-EGFR, as well as a slight inhibition of p-Akt. In contrast, the modulation of p-Erk1/2 by ZD6474 was evident in HT-29 cells only.

Another aspect we considered was the effect on the expression of the tumour suppressor PTEN. Unlike Nagata, who reported PTEN modulation after 1 hour of exposure to the anti-ErbB2 antibody, trastuzumab^[35], no appreciable modulation of this protein was observed in our study after short or long, continuous or alternate drug exposures. Our conclusion was that 14 d of drug exposure may be long enough for recovery of the possibly transient and short-term modulation of this gene potentially occurring in the first few hours after treatment with TK inhibitors.

Tyrosine kinase inhibitors have shown the potential to modulate cytotoxic drug resistance, through an interaction

with ABCG2 and Pgp^[18,19]. In our study, ABCG2, which is involved in camptothecin resistance^[20], was detectable in both HT-29 and LoVo cells while Pgp, which is involved in anthracycline resistance, was not detectable in either cell line. The increased ABCG2 expression, following exposure to gefitinib or ZD6474, was cell line-specific. The HT-29 cells showed a progressive exposure-related increase of 100%-150%, while expression in the LoVo cells was unaffected. The increased expression of ABCG2 in HT-29 cells was schedule-dependent, and it was higher with continuous than with intermittent incubation. Our results seem to be in contrast with those obtained by Nakamura^[19] but the experimental conditions used were completely different. Nakamura used a short time (15 min) to show a gefitinib-dependent increase in topotecan accumulation in transfectant cells, overexpressing ABCG2, and 4 d of gefitinib plus cytotoxic drugs exposure to led to reversal of drug resistance. In our experiments the ability of gefitinib to enhance ABCG2 expression proved to be a late effect of the drug and may account for the antagonism between Topoisomerase-I inhibitors and TK-inhibitors in HT-29 cells induced by a pre-exposure to gefitinib for 5 d^[13]. Moreover, the cell-line-specific effects of gefitinib and ZD6474 on drug-related proteins may provide an explanation for the different results. Wakeling and Ciardiello obtained^[30,31] when analyzing the onset of drug resistance *in vivo* after long-term drug intake.

In conclusion, our investigation studied, in an *in vitro* model of colon cancer, some crucial points related to the clinical use of these TK inhibitor drugs. Evidence has emerged that long term use (maximum 14 d) of these drugs does not lead to a loss of cell activity and intermittent or continuous exposures to these drugs do not produce significantly different toxic effects. The main signal transduction steps for TK receptor pathways were also studied and the findings demonstrated that the effects of these drugs may be highly cell-line-specific (HT-29 *vs* Lovo), drug-dependent (gefitinib *vs* ZD6474) and schedule-related (continuous *vs* intermittent), thus indicating that a predictive factor for these drugs cannot be easily identified or broadly applicable. The data concerning the modulation of drug resistance related proteins in tumour cells treated with long-term drug exposure are even more interesting. ABCG2 expression was shown to be induced by these drugs in a cell-line-specific and schedule-dependent manner. This evidence should suggest caution in choosing exposure times when combining these drugs with Topoisomerase-I inhibitors in particular. The findings of this study can be instrumental in the implementation of future *in vitro* and clinical studies on these drugs.

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COLORECTAL CANCER

BRAF, K-ras and BAT26 mutations in colorectal polyps and stool

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Abstract

AIM: To assess the feasibility of using BRAF, K-ras and BAT26 genes as stool-based molecular markers for detection of colorectal adenomas and hyperplastic polyps (HPs).

METHODS: We applied PCR-SSCP and direct sequencing to detect BRAF mutations of polyps and paired stool samples. Primer-mediated restriction fragment length polymorphism (RFLP) analysis and mutant-enriched PCR were used in detection of K-ras mutations of polyp tissues and paired stool samples respectively. BAT26, a microsatellite instability marker was examined by detection of small unstable alleles in a poly (A) repeat.

RESULTS: No genetic alterations were detected in the 36 colonoscopically normal patients in either tissues or stools. BRAF, K-ras and BAT26 mutations were found in 4 (16%), 10 (40%) and 3 (12%) of 25 adenoma tissues and among them, 75%, 80% and 100% of patients were observed to contain the same mutations in their corresponding stool samples. In HPs, mutations of BRAF and K-ras were detected in the tumor DNA of 2 (11.1%) and 8 (33.3%) of 18 patients respectively, all of whom had identical alterations in their stools. Taken together, the three genetic markers detected 15 (60%) of 25 adenomas and 8 (44.4%) of 18 HPs. The sensitivity of stool detection was 80% for adenomas and 100% for HPs with an overall specificity of 92% for adenomas and 100% for HPs.

CONCLUSION: BRAF, K-ras and BAT26 genes have the potential to be molecular markers for colorectal adenomas and HPs, and can be used as non-invasive screening markers for colorectal polyps.

INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignancies in the world and it is a disease that can be easily cured either by surgery or by endoscopic excision when diagnosed at an early stage^[1]. It has been generally accepted that a majority of CRC develop through a well-defined adenoma-carcinoma sequence in which multiple genetic changes are involved in this pathway that is known as chromosomal instability pathway. K-ras gene is known to play an important role along this pathway in transitioning from early to intermediate adenomas^[2]. However, increasing evidence accumulates that a subset of CRC arises *via* the hyperplastic polyp (HP)-serrated adenoma (SA)-carcinoma sequence that is associated closely with microsatellite instability (MSI) positive colorectal carcinomas^[3-5]. These polyps are usually large and/or multiple and/or located in the proximal colon^[6,7]. APC mutation is not involved in this pathway whereas K-ras and BRAF mutations are frequently observed in these polyps with MSI^[8,9]. Both BRAF and K-ras are proto-oncogenes that interact in tandem in the RAS-RAF-MEK-ERK-MAP kinase signaling pathway, which plays an important role in the control of cell differentiation, proliferation, survival, and apoptosis^[10].

HPs have long been regarded as safe lesions without neoplastic potential^[11]. However, this view has been changed due to the discovery of the HP-SA-carcinoma sequence. Now it is believed that adenomas as well as some types of HPs are precursors of sporadic CRCs that may eventually develop into adenocarcinomas^[2,5]. So detection and surveillance of these premalignant polyps may be of great importance in reducing the incidence of colorectal cancer. BRAF and K-ras mutations have been frequently observed in both adenomas and HPs^[8-10]. This prompted us to detect colon polyps through analysis of these genes in stool DNA. In this study, we detected mutational activation of the K-ras and BRAF genes and along with an MSI marker, BAT26 in patients with adenomas or HPs, and

performed a pair comparison between tumor tissue and stool sample in individual patients to assess the feasibility of using these genes as molecular markers for colon polyps.

MATERIALS AND METHODS

Patients and samples

We recruited 79 patients who underwent colonoscopy for various reasons at the 2nd Affiliated Hospital of Harbin Medical University from June 2004 to March 2005. These included 36 control patients without neoplasms, 18 patients with hyperplastic polyps and 25 patients with adenomas. Diagnosis was histologically confirmed. The mean age of control patients was 49.6 years compared with 51.6 years for patients with HPs and 56.5 years for patients with adenomas. Of the 43 polyps, 34 were from males and 9 were from females. Twenty-seven were from the left colon and 16 were from the right colon. The size of these polyps ranged from 3 to 50 mm in maximal dimension (mean = 5.28 mm for HPs and 18.7 mm for adenomas). Patients who had familial adenomatous polyposis or hereditary nonpolyposis colon cancer, and inflammatory bowel diseases were excluded.

Fresh stool specimens were collected from patients prior to colonoscopy. All the patients were given detailed oral and written instructions for stool collection. Stool samples were frozen immediately at -20°C after collection and transferred to -80°C for permanent storage within 24 h.

With the informed consent of all patients and approval of the ethics committee, paired bioptic polyp tissues were obtained during polypectomy. One part of the tumor was snap frozen in liquid nitrogen and stored at -80°C until the extraction of nucleic acids. Another part was fixed in formalin and paraffin-embedded for diagnosis.

DNA extraction

DNA was isolated from stool samples by means of the QIAamp DNA stool mini kit (QIAGEN, USA) and from snap frozen tissues using Trizol reagent (Invitrogen, Life Technologies, USA) according to the manufacturer's instructions.

Mutational analysis and sequencing of the BRAF gene

To detect possible sequence alterations in BRAF, we performed nonisotopic single-strand conformational polymorphism (SSCP) analysis. The complete sequence of exon 15 of the BRAF gene was amplified from 50 ng of genomic DNA using the primers described previously^[9]. PCR was carried out for 40 cycles with initial denaturation at 95°C for 5 min, followed by 95°C for 40 s, 55°C for 40 s, and 72°C for 40 s in a reaction volume of 25 µL. The PCR products were then separated on 8% nondenaturing polyacrylamide gels (29:1) with or without 5% glycerol, and electrophoresis of the gels was carried out at 4°C-8°C. The gels were then fixed in 10% acetic acid, silver stained in a freshly prepared silver nitrate (0.1%) and developed in 3% sodium carbonate with 0.05% formaldehyde and 0.2% sodium thiosulphate.

All PCR products showing mobility shifts on SSCP were reamplified and purified, and evaluated by direct

sequencing with an ABI 3700 DNA sequencer (Applied Biosystems).

Primer-mediated RFLP analysis

The mutations at codons 12 and 13 of K-ras gene in tumor samples were screened by primer-mediated restriction fragment length polymorphism (RFLP) analysis as described^[12]. Human placental DNA (Sigma, USA) was used as a wild type control. Colorectal carcinoma DNA with known mutations at K-ras codon 12 (GGT to GTT) and at codon 13 (GGC to GAC) was used as mutant controls.

Mutant-enriched PCR

Mutant-enriched PCR was used to analyze the mutational status of the K-ras gene in stool samples. The procedure was the same as described^[12] with the only exception that 5 U of BstNI or Bgl I (NEB, Beijing) was used for the digestion, and the reaction was completed overnight. Final products were separated on 4% agarose gel and visualized by ethidium bromide staining. The same positive and negative controls were used as in PCR-RFLP analysis.

MSI analysis

The MSI marker used was BAT-26, a mononucleotide repeat that by itself is a very good measure of generalized instability. PCR reactions were performed with the specific primers reported previously^[13]. PCR products were loaded on 8% polyacrylamide/ 7 mol/L urea DNA-denaturing sequencing gels and silver stained as described above.

Statistical analysis

Differences between groups were assessed by χ^2 test and Fisher's exact test. All P values were two sided. Factors with $P < 0.05$ were considered statistically significant. The 95% confidence intervals (CI) were determined based on the exact binomial distribution.

RESULTS

Twenty-five sporadic adenomas and 18 hyperplastic polyps were collected and analyzed for the alterations of K-ras, BRAF and BAT26 genes. The median age of the patients with HPs was 56 years (range: 28-70) compared with 61.8 years for patients with adenomas (range: 40-74). Thirty-six stool samples from control patients were also detected.

Adenomas

Mutations of exon 15 of BRAF and codons 12, 13 of K-ras genes were analyzed, as these cover most of the mutation hot spots known of the two genes. The alterations of BRAF, K-ras and BAT26 genes in adenomas and their relationship with clinico-pathological characteristics are shown in Table 1.

Of 25 tumor samples detected, 15 [60%, 95% CI: 39%-79%] were found to have at least one alteration in BRAF, K-ras or BAT26 genes. BRAF mutations were identified in 4 (16%, 95% CI: 5%-36%) cases, all at nucleotide position 1799 with T-A transversions (V599E) as confirmed by direct sequencing (Table 1, Figure 1). No significant correlation was found between BRAF mutations with

Table 1 Genetic analysis of adenomas and HPs compared with clinical-pathological characteristics

	<i>n</i>	BRAF <i>n</i> (%)	K-ras <i>n</i> (%)	BAT26 <i>n</i> (%)	All markers <i>n</i> (%)
Adenomas	25	4 (16)	10 (40)	3 (12)	15 (60)
Gender					
M	19	3 (15.8)	7 (36.8)	2 (10.5)	
F	6	1 (16.7)	3 (50)	1 (16.7)	
Age (yr)					
< 60	14	3 (21.4)	7 (50)	3 (21.4)	
≥ 60	11	1 (9.1)	3 (27.3)	0	
Location					
Distal	16	2 (12.5)	8 (50)	1 (6.3)	
Proximal	9	2 (22.2)	2 (22.2)	2 (22.2)	
Size (mm)					
< 10	5	1 (20)	2 (40)	0	
≥ 10	20	3 (15)	8 (40)	3 (15)	
HPs	18	2 (11.1)	6 (33.3)	0	8 (44.4)
Gender					
M	15	2 (13.3)	5 (33.3)	0	
F	3	0	1 (33.3)	0	
Age (yr)					
< 60	12	2 (16.7)	5 (41.7)	0	
≥ 60	6	0	1 (16.7)	0	
Location					
Distal	11	1 (9.1)	4 (36.4)	0	
Proximal	7	1 (14.3)	2 (28.6)	0	
Size (mm)					
< 10	15	0	4 (26.7)	0	
≥ 10	3	2 (66.7) ¹	2 (66.7)	0	
Number of HP					
SP	4	1 (25)	1 (25)	0	
MP	14	1 (7.1)	5 (35.5)	0	

Group HPs included the individuals with only hyperplastic polyps. If an individual had multiple polyps and if any one of the polyps exceeded 10 mm in diameter, it was regarded as ≥ 10 mm in size. SP: Single polyp; MP: Multiple polyps. Proximal tumors were defined as cecum through transverse colon; tumors in the splenic flexure, descending, and sigmoid colon were defined as distal. ¹Fisher's exact test, *P* = 0.01.

Table 2 BRAF mutations in colorectal adenomas and HPs with BAT26 status

	No of samples	No of BRAF mutation (%)
Adenomas	25	4 (16)
BAT26 (+)	3	2 (66.7) ¹
BAT26 (-)	22	1 (4.5)
HPs	18	2 (11.1)
BAT26 (+)	0	0
BAT26 (-)	18	2 (11.1)

¹Results from Fisher' exact test, BAT26 (+) vs BAT26 (-), *P* = 0.028.

patient's gender, age, location of tumor and tumor size (Table 1). The identical V599E mutations of the BRAF gene were also observed in 3/25 (12%, 95% CI: 3%-31%) of fecal samples with a 75% agreement between tumor and stool.

In total, 3/25 (12%, CI: 3%-31%) of tumor DNAs showed BAT26 alterations (Table 1) and the same mutations were also observed in all the 3 paired stool samples. Two of the 3 samples with a BAT26 alteration also harbored a BRAF mutation that showed a close

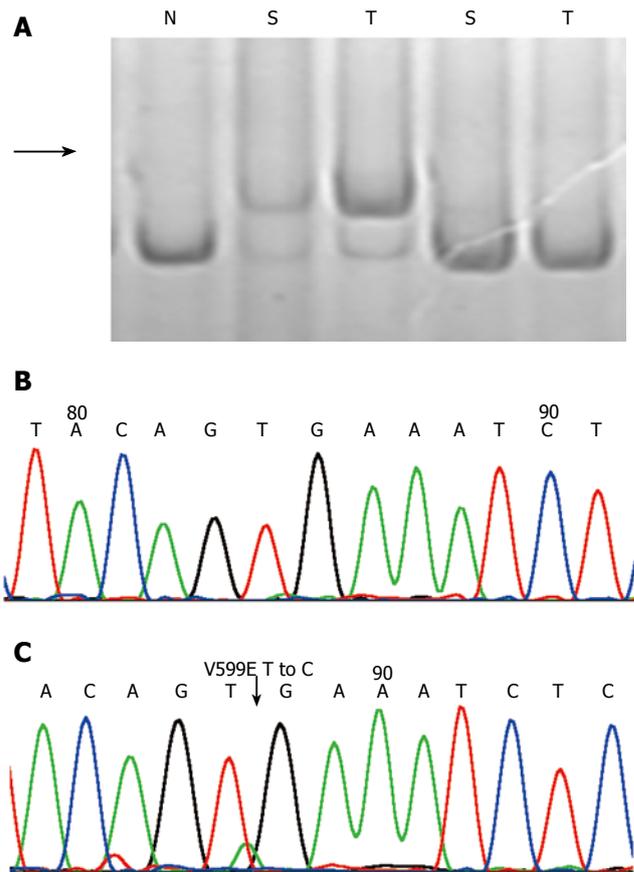


Figure 1 Example of BRAF mutation analysis in adenoma tissues (T) and paired stool (S) samples. **A:** SSCP analysis; **B:** Direct sequencing showing the wild type BRAF; **C:** BRAF V599E mutation; N: normal. Arrows indicate the new band (A) and the mutation site (C).

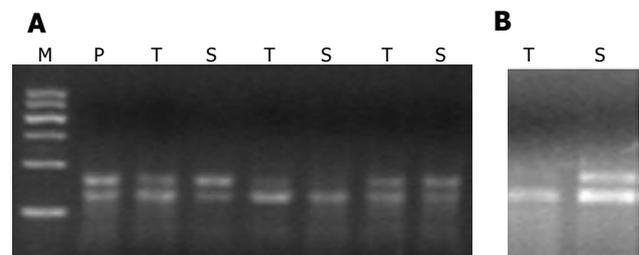


Figure 2 Analysis of K-ras mutations at codon 12 (A) and codon 13 (B) in adenoma tissues (T) and paired stool (S) samples. Fragments of 157 bp indicate mutations, and fragments of 128 bp(A) or 125 bp (B) respectively represent wild-type alleles. M: 100 bp ladder marker; P: Positive control; B shows the result that a mutation at codon 13 of K-ras in stool DNA was not observed in tumor counterpart.

relationship between BRAF mutation and MSI (66.7% vs 4.5%, *P* < 0.05, Table 2). All 3 BAT26 positive samples were found in patients younger than 60 year and in adenomas larger than 10 mm in size but of no statistical significance. Also no significant relationship was found with respect to patient's gender and tumor site.

Among 25 tumor samples analyzed for K-ras mutations at codons 12 and 13, 10 (40%; 95% CI: 21%-61%) samples were found to carry a mutation, 8 of which were at codon 12 and 2 at codon 13 (Table 1, Figure 2). Mutations of the K-ras gene in corresponding stool samples were detected by mutant-enriched PCR^[12]. Of 25 stool samples

analyzed, K-ras alterations were observed in 9 (36%, 95% CI: 18%-58%) patients, 7 at codon 12 and 2 at codon 13. Eight of 10 stool DNAs had a mutation that matched exactly with the results of the corresponding tumor analysis (sensitivity: 80%, 95% CI: 44%-97%) whereas there was one case in which a K-ras mutation at codon 13 was observed in stool DNA but could not be detected in the corresponding tumor (specificity: 94%, 95% CI: 71%-100%, Figure 2). None of the tumor or stool samples with V599E carried a K-ras mutation.

To sum up, the three genetic markers detected 13/25 (52%) of stool samples of adenoma patients. The overall sensitivity of stool analysis using these three genes was 80% (12/15, 95% CI: 52%-96%) with a specificity of 92% (12/13, 95% CI: 64%-100%).

Hyperplastic polyps and normal controls

Eight (44.4%, 95% CI: 22%-69%) of the 18 HPs were found positive in either the BRAF or K-ras gene while no alterations were found in the BAT26 gene (Table 1). In corresponding stool samples, the same mutations were identified in all the 8 samples whose tumors contained the alterations with a 100% agreement between tumors and stools. No alterations were found in the other 10 samples. The specificity was also 100%. BRAF mutations were more common in polyps larger than 10 mm in size ($P < 0.05$, Table 1). BRAF and K-ras mutations were also mutually exclusive. No alterations were found in any of the three markers tested in 36 control DNAs of fecal origin.

DISCUSSION

In this study, we assessed the use of genetic alterations of BRAF, K-ras and BAT26 genes as stool-based molecular markers for colon adenomas. To our knowledge, this is the first time to show the results of stool-based gene detection of colorectal hyperplastic polyps.

We identified 16% of BRAF mutations and 40% of K-ras mutations in adenoma tissues. The results are similar to those reported previously^[10]. However, in HPs, we observed a relatively lower incidence of BRAF mutations and a higher incidence of K-ras mutations than other studies^[8,9]. Chan^[8] reported a 36% BRAF mutation rate in HPs and Yang^[9] reported an even higher mutation rate of 69.6%, but our result was only 11.1%. The incidence of K-ras mutations was 18% and 20% respectively by Chan^[8] and Yang^[9] but ours was 33.3%. This difference may be due to the small sample numbers in our study. The only form of BRAF mutation observed in our study was V599E. It is the most common mutation identified to date^[8-10]. This missense mutation has been proven to maximally activate kinase activity of the BRAF protein and mutated forms of BRAF can transform NIH3T3 cells^[14]. It is obvious that this variant has a strong functional selection for growth advantage. The high incidence of mutually exclusive mutations of BRAF and K-ras in both adenomas and HPs supports the previous hypothesis that activations of both genes are an early event in tumorigenesis of CRC^[8-10].

BAT26 alteration was observed in 3/25 (12%) of adenomas, two of which also harbored a BRAF mutation. This close relationship between BRAF and MSI status

agrees well with the findings in sporadic CRC^[10]. By contrast, no BAT26 alteration was found in HPs. High level MSI was more often found in tumors located in the right colon, and most of our HPs were located in the left colon. However, several other studies have also reported a low MSI in HPs and serrated adenomas^[15].

It is interesting to note that although not significant, in our study, patients with > 60 years of age had a tendency towards a lower prevalence of any mutation in both the adenoma and HPs groups. This may be due to the small sample numbers detected in our study, and larger sample analysis is needed to prove this phenomenon.

Based on our findings in the adenomas and HPs, we tried to use these three genes as stool based molecular markers for colon adenomas and HPs. Of the 15 adenomas that showed positive results, 12 (80%) samples have been detected to be positive in corresponding stool DNA, with a specificity of 92%. This result is quite comparable with the findings reported previously^[16-20], in which DNA panel targeted mutations at K-ras, APC and p53 genes as well as BAT26 and long DNAs were used. But our detection seemed to be more convenient. First, mutation at the BRAF V599E hotspot is relatively simple to detect using SSCP and the sequencing method used in the present work (Figure 1). Second, the entire sequence of BRAF exon 15 is rather short, about 250 bp long, which can be amplified effortlessly, even in the stool DNA (data not shown).

In our study, we have detected a K-ras mutation in one stool DNA that could not be observed in corresponding adenoma tissue DNA. This is not caused by the different detection method we used in stools and tissues, because we analyzed this tissue DNA again using mutant-enriched PCR as in stool DNA, but still no mutation was found. This disparity might be due to other polyps present in the colon of this patient which we failed to detect through colonoscopy or because other neoplasms outside the colon were present in this patient that we did not know, since K-ras mutations have been detected in patients with pancreatic diseases^[21].

In addition to adenomas, we also analyzed the DNA panel in patients with HPs. Our results showed that 44.4% of stool samples carried a mutation of either the BRAF or K-ras gene. All the mutations found in HPs could also be detected in corresponding stool DNA with a specificity of 100%. The results indicate that the mutations in HPs, even if they come from a very small tumor, can be detected in the stool DNA. It also proves that the technique of stool-based DNA detection for colorectal tumors is very sensitive.

Considering the small sample numbers and selected populations of symptomatic patients in our study, large investigations of fecal DNA analysis using these genes in asymptomatic populations are needed. In addition, recent investigations have shown that methylation of the hMLH1 is very common in HPs with MSI^[5]. Thus, the combinations of genetic markers with epigenetic methylated genes will probably increase the sensitivity of the colorectal polyps detection rate.

In summary, our data indicate that mutations of BRAF, K-ras and BAT26 genes in adenomas and HPs are frequent and can be detected in corresponding stool samples. They

can be used as stool based genetic markers for detection of colon polyps.

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Synergistic effect of a novel oxymatrine-baicalin combination against hepatitis B virus replication, α smooth muscle actin expression and type I collagen synthesis *in vitro*

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Abstract

AIM: To study the effect of oxymatrine-baicalin combination (OB) against HBV replication in 2.2.15 cells and α smooth muscle actin (α SMA) expression, type I, collagen synthesis in HSC-T6 cells.

METHODS: The 2.2.15 cells and HSC-T6 cells were cultured and treated respectively. HBsAg and HBeAg in the culture supernatants were detected by ELISA and HBV DNA levels were determined by fluorescence quantitative PCR. Total RNA was extracted from HSC-T6 cells and reverse transcribed into cDNA. The cDNAs were amplified by PCR and the quantities were expressed in proportion to β actin. The total cellular proteins extracted from HSC-T6 cells were separated by electrophoresis. Resolved proteins were electrophoretically transferred to nitrocellulose membrane. Protein bands were revealed and the quantities were corrected by β actin.

RESULTS: In the 2.2.15 cell culture system, the inhibitory rate against secretion of HBsAg and HBeAg in the OB group was significantly stronger than that in the oxymatrine group (HBsAg, $P = 0.043$; HBeAg, $P = 0.026$; respectively); HBV DNA level in the OB group was significantly lower than that in the oxymatrine group ($P = 0.041$). In HSC-T6 cells the mRNA and protein expression levels of α SMA in the OB group were significantly lower as compared with those in the oxymatrine group (mRNA, $P = 0.013$; protein, $P = 0.042$; respectively); The mRNA and protein expression levels of type I collagen in the OB group were significantly lower as compared with those in the oxymatrine group (mRNA, $P < 0.01$; protein, $P < 0.01$; respectively).

CONCLUSION: OB combination has a better effect

against HBV replication in 2.2.15 cells and is more effective against α SMA expression and type I collagen synthesis in HSC-T6 cells than oxymatrine *in vitro*.

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Key words: 2.2.15 cells; HSC-T6 cells; Oxymatrine; Baicalin; Hepatitis B virus; α smooth muscle actin; Type I collagen

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INTRODUCTION

The human hepatitis B virus (HBV) belongs to the family of small DNA hepadnaviruses. HBV infection is a major cause of chronic hepatitis, hepatic fibrosis, liver cirrhosis, and hepatocellular carcinoma and results in one million deaths annually^[1]. In China there are about thirty million patients suffering from chronic hepatitis B. Hepatic fibrosis is a kind of compensating and healing response to liver injuries. And hepatic fibrogenesis has been known to be a gradual and dynamic process associated with the continuous deposition and resorption of connective tissues and collagens^[2,3]. During the hepatic fibrogenesis process, hepatic stellate cells (HSC, formerly termed as lipocytes, Ito cells or fat-storing cells) play a central role based on their ability to undergo activation following liver injury of any cause. HSC has been recognized to be responsible for most of the excess extracellular matrix (ECM) observed in chronic hepatic fibrosis^[4].

For a few decades, interferon α (IFN α) has been the only approved therapy for chronic HBV infection around the world. But its efficacy is not satisfactory and associated with some adverse reactions such as influenza-like syndrome, leukocyte and platelet decrease^[5]. Recently, lamivudine has been the first nucleotide analog approved for treating chronic HBV infection in many regions of the world; but its efficacy is just similar to IFN α and prolonged administration is associated with drug

resistance and virus variation, which could lead to severe consequences including liver failure^[6] and even death^[7]. Up to now, the treatment of HBV is still a difficult problem. Therefore, it is necessary to develop novel drugs and treatment methods.

Oxymatrine is a kind of alkaloid extraction derived from a Chinese herb *Sophora flavescens* Ait^[2,3]. It has been widely used for treating viral hepatitis B and C and hepatic fibrosis in recent years in China^[8,9]. But just like other anti-HBV drugs, how to improve its efficacy against HBV and hepatic fibrosis is still an urgent challenge in clinical practice. On the basis of our previous findings^[10], in the present study, the 2.2.15 cells^[11], the hepatoblastoma cell line HepG2 transfected with cloned hepatitis B virus DNA, and the rat HSC-T6 cells^[12], an immortalized rat hepatic stellate cell line, were cultured respectively, and the effects of a novel oxymatrine-baicalin combination^[10] (OB) against HBV replication and α smooth muscle actin (α SMA) expression, type I collagen synthesis *in vitro* were evaluated.

MATERIALS AND METHODS

Drugs, reagents and instruments

Oxymatrine, and OB combination were prepared and provided by Shanghai Kairuisi Biotech Co. Ltd. according to our applied China National Invention Patent^[13]. Dulbecco's modified Eagle's medium (DMEM) and modified Eagle's medium (MEM) culture media were the products of Gibco BRL. Fetal bovine sera (FBS) were purchased from Hyclone (Logan, Utah, USA). G418 was purchased from Shanghai Jiebeisi Gene-Tech Co. Ltd. L-glutamine and EDTA were supplied by Shanghai Shisheng Biotech Co. Ltd. The ELISA kits for HBsAg (hepatitis B surface antigen) and HBeAg (hepatitis B e antigen) kits were ordered from Huamei Biotech Co. Ltd. The penicillin and streptomycin were the products of Shanghai Xianfeng Pharmacological Co.

The HBV DNA (deoxyribonucleic acid) PCR (polymerase chain reaction)-fluorescence quantitative diagnostic kit was purchased from Shanghai Kehua Bio-engineering Co. Ltd. Trizol reagent was the product of Invitrogen. DNA marker was purchased from Tiangen Co. The cDNA synthesis kit and PCR master mix kit were purchased from Fermentas Co. The specific primers were synthesized by Shanghai Shenggong Co. The rabbit anti-rat type I collagen polyclonal antibody was the product of Merck. The mouse anti-rat α SMA mAb and anti-rat β actin mAb were purchased from Sigma (St. Louis, MO). The DC protein quantification kit was the product of Bio-Rad (Bio-Rad Laboratories, Hercules, CA, USA). Enhanced chemiluminescence reagents were purchased from Pierce. The other reagents routinely used in our laboratory were all of analytical grade.

Cell culture flasks and plates were the products of Corning Inc (Corning, NY, USA). CO₂ culture hood and Healforce hood were the products of Heraeus Co. Labsystems Multiskan MS Microplate Reader was made in Finland. XDS-1B invert phase-contrast microscope was purchased from Chongqin Guangdian Co. The LightCycler fluorescence PCR system was the product of Roche Co.

The Rotor-Gene RG-3000 PCR machine was the product of Gene Co. The Furi FR-980 image analysis system was provided by Shanghai Furi Co. Western blot instrument was produced by Bio-Rad.

Cell culture and treatment

The 2.2.15 cell line was purchased from the Department of Microbiology, Tianjin Medical University, China. The 2.2.15 cells were cultured in complete MEM culture media (supplemented with 100 mL/L FBS, 3.8 g/L L-glutamine, 0.38 g/L G418, 50 ku/L penicillin and streptomycin, pH 7.0) at 37°C in 50 mL/L CO₂, 950 mL/L air. When cells were in the logarithmic growth phase, they were trypsinized and seeded. Experiments were performed when cells reached 80% confluence. The cells were supplemented with oxymatrine or OB combination and incubated for an additional 4 d. The working concentration of oxymatrine was 1 g/L and the OB combination contained 1 g/L oxymatrine^[10] and a specific concentration of baicalin^[13] according to our previous findings. At the above concentrations these drugs have no cytotoxic effect to cells by MTT colorimetric assay^[10]. Then the cell culture medium was collected according to the experimental protocol. All experiments were performed in duplicate or triplicate samples.

HSC-T6 cells were kindly provided by Professor Scott Friedman (Liver Center Laboratory, San Francisco General Hospital, USA) and has been stored and passaged routinely in our institute. HSC-T6 cells were seeded in DMEM with 100 mL/L FBS at 37°C, in 50 mL/L CO₂, 950 mL/L air. When cells were in the logarithmic growth phase, they were trypsinized and seeded into 60 mm culture plates. Experiments were performed when cells reached 80% confluence. Each plate was supplemented with 2 mL culture media with oxymatrine or OB combination and incubated for an additional 24 h. Then the cells were harvested according to the experimental protocol. All experiments were performed in duplicate or triplicate samples.

Measurement of HBV antigens secretion in supernatants of 2.2.15 cells by ELISA

The 2.2.15 cells were incubated at a density of 1×10^9 /L in 1 L MEM medium containing 100 mL/L FBS. After 24 h incubation, the 2.2.15 cells were treated with oxymatrine or OB combination respectively. Cells were cultured in the presence of drugs for 4 d. Then the supernatants were collected and stored at -20°C until measurement. The HBsAg and HBeAg were detected simultaneously by ELISA kits according to the manufacturer's instruction, and the inhibitory rates for HBsAg and HBeAg were calculated respectively.

Measurement of HBV DNA replication in supernatants of 2.2.15 cells by fluorescence quantitative PCR

The HBV DNA level in the supernatants post-drug treatment was determined by the HBV DNA PCR-quantitative diagnostic kit using the Roche LightCycler system according to the manufacturer's protocol. One hundred microliter culture medium was added to 100 μ L sample reagent A, and then centrifuged at 13000 r/min

for 10 min. The supernatants were discarded and 25 μ L sample reagent B was added. After centrifugation for a few seconds, the mixture was heated at 100°C for 10 min. Then 2 μ L of the supernatants after centrifugation at 13 000 r/min for 10 min was mixed with 18 μ L PCR reaction reagent, and analyzed at Channel F1 by the LightCycler system.

Determination of α SMA and type I collagen mRNA levels by semi-quantitative RT-PCR

Total RNA was extracted from HSC-T6 cells by Trizol reagent following the protocols provided by the manufacturer. The integrity of total RNA was confirmed by the denaturing formaldehyde agarose gel electrophoresis. The quantity and purity of RNA were detected by determining absorbance at 260/280 nm using a spectrophotometer. Total RNA was reverse transcribed into complementary DNA (cDNA) using the cDNA synthesis kit. The reverse-transcription (RT) reaction mixtures were amplified by semi-quantitative PCR using specific primers for the target genes and β actin. The primers used are as follows: Type I collagen, forward: 5'-TAC AGC ACG CTT GTG GAT G-3', reverse: 5'-TTG AGT TTG GGT TGT TGG TC-3', target fragment length 259 bp; α SMA, forward: 5'-CCG ACC GAA TGC AGA AGG-3', reverse: 5'-ACA GAG TAT TTG CGC TCC GGA-3', target fragment length 88 bp; β actin, forward: 5'-TGA CGA GGC CCA GAG CAA GA-3', reverse: 5'-ATG GGC ACA GTG TGG GTG AC-3', target fragment length 330 bp. The 20 μ L reaction mixture consisted of the corresponding primers, 1 U of Taq polymerase, 50 μ mol/L of each of the four dNTP, 1 \times PCR buffer supplemented with 2.5 mmol/L MgCl₂. After an initial melting time of 5 min at 94°C, the mixtures were subjected to 35 PCR cycles, consisting of denaturation for 1 min at 94°C, primer annealing for 1 min at 55°C, and primer extension for 1 min at 72°C, with a final extension time of 10 min at 72°C. Quantification of the final products was performed by electrophoresis in 30 g/L agarose gel using the Furi FR-980 image analysis system. The number of mRNA molecules was expressed in proportion to the number of internal control β actin in the same sample as described before^[14].

Determination of α SMA and type I collagen protein levels by Western blot

HSC-T6 cell lysates were prepared from 1×10^7 cells by dissolving cell pellets in 100-200 μ L lysis buffer (150 mmol/L NaCl, 10 g/L tergitol NP-40, 5 g/L sodium deoxycholate, 0.1 g/L sodium dodecyl sulfate, 50 mmol/L Tris, and a protease inhibitor cocktail, pH7.5). Lysates were centrifuged at 4°C, 12 000 r/min for 15 min, then the supernatants were collected and stored at -70°C until detection. Protein content was analyzed by DC protein assay and at 690 nm wavelength. Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (0.31 mL 2 mol/L Tris buffer (pH6.8), 20 g/L SDS, 100 mL/L glycerol, 2.4 g/L DTT, 50 g/L β -mercaptoethanol, 0.02 g/L bromophenol blue) was added to the lysates. After mixing the lysates were heated to 100°C for 5 min, and 50 μ g sample protein was loaded into

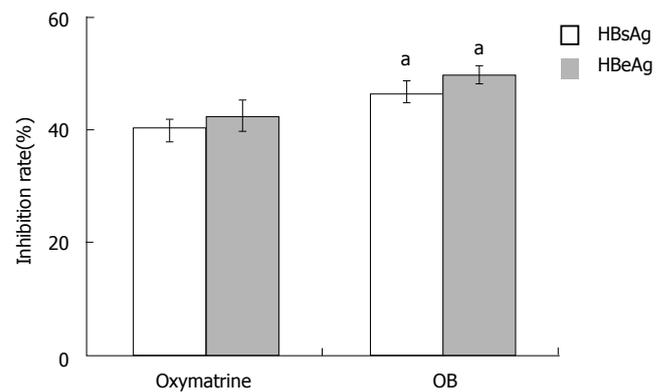


Figure 1 The inhibition rate of OB on HBsAg and HBeAg secretion in the 2.2.15 cell culture system. $n = 3$, mean \pm SD. ^a $P < 0.05$ vs oxymatrine.

each well of 100 g/L SDS-PAGE gel. Resolved proteins were electrophoretically transferred to nitrocellulose membrane and blocked with 50 mL/L non-fatty milk. Then the primary antibodies (mouse anti-rat β actin antibody, 1:5000 dilution; mouse anti-rat α SMA antibody, 1:5000 dilution, rabbit anti-rat type I collagen antibody, 1:200 dilutions) in 50 mL/L non-fatty TTBS solution (1.21 g/L Tris, 9 g/L NaCl, 1 g/L Tween-20, pH7.5) were added respectively. After incubation at 4°C overnight the blots were washed, then membranes were incubated with corresponding horseradish peroxidase-conjugated secondary antibodies for 1 h. Protein bands were revealed by the ECL kit according to the manufacturer's protocol and the Furi FR-980 image analysis system was used for quantitative analysis of the blots. β actin protein was used as the internal control as described before^[15].

Statistical analysis

All results were expressed as mean \pm SD. Comparisons were analyzed by one-way ANOVA using the SPSS 10.0 statistical package. Differences were considered statistically significant if the $P < 0.05$.

RESULTS

Effect of OB on HBV antigen secretion in 2.2.15 cells

After incubation for 4 d, HBsAg and HBeAg secretion in the culture medium was determined by ELISA. As shown in Figure 1, both OB and oxymatrine had remarkable inhibitory effects on secretion of HBsAg and HBeAg in the 2.2.15 cells, the inhibitory rate in the OB group was significantly stronger than that in oxymatrine group (HBsAg, $P = 0.043$; HBeAg, $P = 0.026$; respectively). And the inhibitory rate on HBeAg in both groups exceeded that of HBsAg.

Effect of OB on HBV DNA replication in 2.2.15 cells

After incubation for four days, the levels of HBV DNA in the culture medium were determined by fluorescence quantitative PCR. As shown in Figure 2, both OB and oxymatrine reduced the HBV DNA level in the supernatant of the 2.2.15 cells, and the HBV DNA level in the OB group was significantly lower than that of the oxymatrine group ($P = 0.041$), indicating that OB inhibited

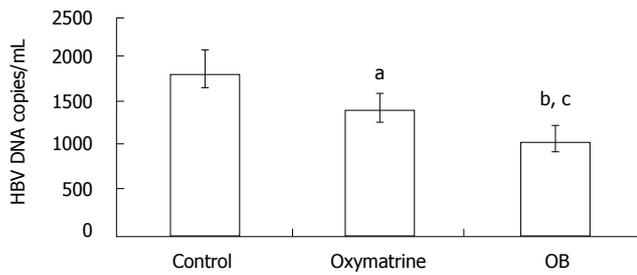


Figure 2 The effect of OB on the HBV DNA level in the 2.2.15 culture system. $n = 3$, mean \pm SD. ^a $P < 0.05$, ^b $P < 0.01$ vs control, ^c $P < 0.05$ vs oxymatrine.

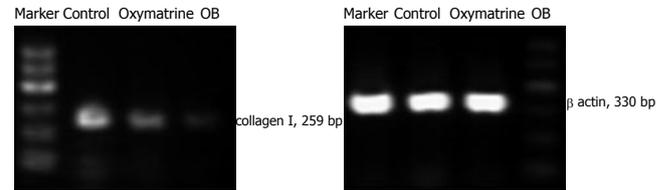
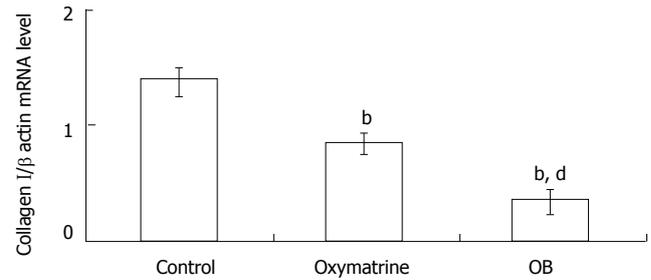


Figure 5 Effect of OB treatment on the Collagen I mRNA level in HSC-T6 cells. The collagen mRNA level was determined by semi quantitative RT-PCR analysis and corrected by the β actin mRNA level. mean \pm SD. ^b $P < 0.01$ vs control, ^d $P < 0.01$ vs oxymatrine.

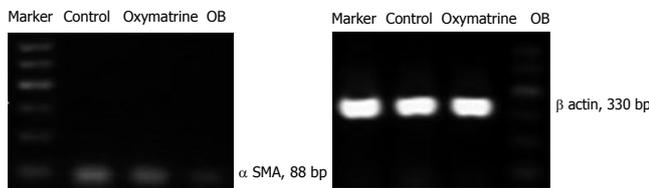
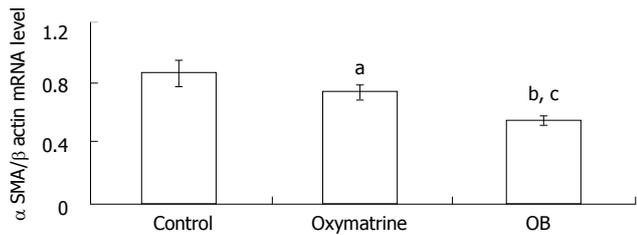


Figure 3 Effect of OB treatment on the α SMA mRNA level in HSC-T6 cells. The α SMA mRNA level was determined by semi quantitative RT-PCR analysis and corrected by β actin mRNA level. $n = 3$, mean \pm SD. ^a $P < 0.05$, ^b $P < 0.01$ vs control, ^c $P < 0.05$ vs oxymatrine.

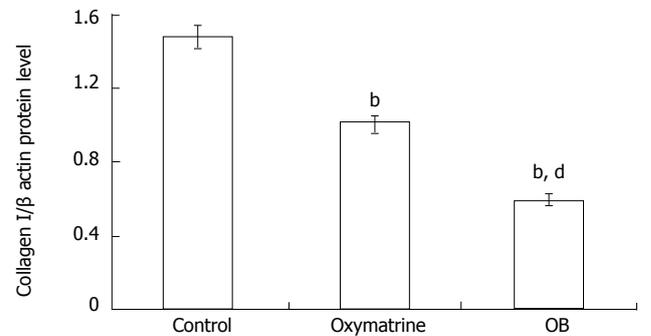


Figure 6 Effect of OB treatment on the collagen I protein level in HSC-T6 cells. The collagen protein level was determined by Western blot analysis and corrected by the β actin protein level. $n = 3$, mean \pm SD. ^b $P < 0.01$ vs control, ^d $P < 0.01$ vs oxymatrine.

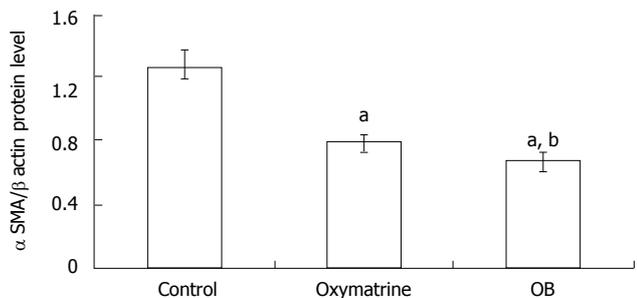


Figure 4 Effect of OB treatment on the α SMA protein level in HSC-T6 cells. The α SMA protein level was determined by Western blot analysis and corrected by β actin protein level. $n = 3$, mean \pm SD. ^a $P < 0.01$ vs control, ^b $P < 0.05$ vs oxymatrine.

HBV DNA replication more effectively than oxymatrine did.

Effect of OB on α SMA expression in HSC-T6 cells

The α SMA mRNA and protein expression levels in HSC-T6 cells were determined by semi-quantitative PCR and Western blot analysis respectively. The β actin mRNA and protein were chosen as internal controls. The results

showed that the mRNA and protein expression levels of α SMA in both the oxymatrine and OB groups were significantly reduced compared with the control group (mRNA, $P = 0.033$ and $P = 0.001$ vs the control; protein, $P < 0.01$ and $P < 0.01$ vs the control; respectively). As shown in Figures 3 and 4, the mRNA and protein expression levels of α SMA in the OB group were significantly lower as compared with those in the oxymatrine group, suggesting that OB reduced the HSC-T6 activation more effectively than oxymatrine (mRNA, $P = 0.013$; protein $P = 0.042$; respectively).

Effect of OB on type I collagen synthesis in HSC-T6 cells

The type I collagen mRNA and protein expression levels were also determined. As shown in Figures 5 and 6, the mRNA and protein expression levels in both the oxymatrine and OB groups were significantly reduced compared with the control group (mRNA, $P < 0.01$ and P

< 0.01; protein, $P < 0.01$ and $P < 0.01$; respectively). The results showed that the mRNA and protein expression levels in the OB group were significantly lower as compared with those in the oxymatrine group, suggesting that the OB combination reduced the synthesis of type I collagen in HSC-T6 cells more effectively than oxymatrine not only at mRNA level but also at the protein level (mRNA, $P < 0.01$; protein, $P < 0.01$; respectively).

DISCUSSION

The World Health Organization places HBV infection among the top 10 causes of death worldwide. It is estimated that there are over 400 million carriers of HBV. At least 20% to 30% of HBsAg carriers will die of complications of chronic liver disease, including cirrhosis and liver cancer. The serious consequences of end-stage liver disease and liver cancer occur in 30% of chronic carriers^[1,5,16]. Investigation of the expression and replication of the HBV genome has been hampered by the lack of an *in vitro* tissue culture system until the 2.2.15 cell line was established^[11]. The medium of these cells contains not only all of the particles present in the serum of infected individuals but also a number of replicative intermediates that probably represent recircularized, ccc, and single-stranded HBV DNA. Various parameters of the replicative cycle can be quantitated in the 2.2.15 culture system, for example, the secretion of HBsAg or HBeAg and the amount of episomal HBV DNA. At present, the 2.2.15 cell line is still the main *in vitro* model used for evaluating the anti-HBV effect of drugs^[10].

Because HBV infection plays a key role in the development of decompensated cirrhosis and hepatocellular carcinoma, HBV patients with serum aminotransferase twice the upper limit of reference value and HBV DNA positive in the blood were advised to receive antiviral agents treatment^[5,8]. Oxymatrine has been used widely in the treatment of chronic liver disease including hepatitis B, hepatitis C and hepatic fibrosis^[2,3,7,8]. As for etiological treatment, oxymatrine could effectively treat chronic viral hepatitis and promote the serum markers of HBV in chronic hepatitis B patients to convert to negative and reduce serum level of ALT^[8,9]. But its efficacy is similar to IFN α , therefore, it is urgent to improve the treatment efficacy of oxymatrine. The present study showed that the inhibitory rate of OB combination against HBV antigens secretion in the supernatant of the 2.2.15 culture system was significantly stronger than that of oxymatrine. In order to elucidate the influence of OB combination on HBV replication, HBV DNA was determined in the supernatants also and the results indicated that OB combination could suppress virus replication and the effect was superior to oxymatrine treatment alone. The pattern of OB combination against HBV is consistent with those reports on oxymatrine elsewhere^[16,17].

The objective of anti-HBV therapy is not only to eradicate the virus but also to prevent the development of hepatic fibrosis. Hepatic fibrosis, a precursor of cirrhosis, is a consequence of severe liver damage that occurs in many patients with chronic liver disease, and involves the abnormal accumulation of ECM^[2,3,9,14]. Liver fibrosis

represents a major healthcare burden worldwide. During hepatic injury, HSCs become active and undergo profound phenotypic changes^[4,18]. HSCs are non-parenchyma cells, located perisinusoidally in the space of Disse. The expression of α SMA is the most important feature of HSC activation. HSC is the primary source of excessive production of ECM. The collagens are the major ECM component of normal and fibrotic livers. In the normal liver the amount of type III collagen is greater than type I collagen, but type I collagen is particularly produced predominantly during fibrogenesis. While the primary HSC cultures are a useful tool for studying hepatic fibrogenesis, their isolation is extremely time-consuming, the yields are modest, and there is considerable preparation-to-preparation variability. The rat HSC-T6 cell line^[12] is constructed through transfecting SV40 into rat HSC and its phenotype is activated HSC. HSC-T6 has the stable phenotype and biochemical characters of activated HSC, expressing myogenic and neural crest cytoskeletal filaments, and the cell line has been a useful tool for studying hepatic fibrogenesis and it is also a reliable cell model for investigating antifibrotic drugs^[19].

In this study the RT-PCR and Western blot analysis results showed that OB combination had a stronger inhibitory effect against the activation of HSC, and this was proven by the significantly lower levels of α SMA mRNA and protein in the OB group than those in the oxymatrine group. Type I collagen is produced predominantly during hepatic fibrogenesis^[4,18]. Inhibiting the secretion of type I collagen not only reduced the ECM component, but also decreased further activation of HSC^[19]. Our results showed that the OB combination could not only inhibited the synthesis of type I collagen at the transcription level but also at the translation level, and these inhibitory effects of the OB combination were superior to those of oxymatrine.

In addition to the anti-HBV effect^[8,16], it was reported that oxymatrine has an effect against liver fibrosis *in vitro*^[15] and *in vivo*^[2,3,17,21], protects animals from fulminant hepatitis^[22] or inflammation^[23]. Baicalin, a flavonoid isolated from the root of *Scutellaria baicalensis* Georgi^[10], has been demonstrated to have multiple biological functions, such as anti-HBV^[9,24], inhibiting HIV infection^[25], inhibiting SARS coronavirus^[26]. Romero^[24] confirmed that baicalin has a moderate ability to reduce HBV production and has no toxic effect on host cells, and this effect against HBV was confirmed by our findings^[10] also. Moreover, it was reported that baicalin^[25] at the noncytotoxic concentrations inhibited both T cell tropic (X4) and monocyte tropic (R5) HIV-1 Env protein mediated fusion with cells expressing CD4/CXCR4 or CD4/CCR5, and the presence of baicalin at the initial stage of HIV-1 viral adsorption blocked the replication of HIV-1, resulting in an early definitive DNA replication cessation. Chen^[27] found that baicalin had antiviral activity against 10 clinical isolates of SARS coronavirus by neutralization tests, which was confirmed by plaque reduction assays. Jang^[28] reported that baicalin had a protective effect against acetaminophen-induced hepatotoxicity in mice and showed that the effects might be due to a block of the bioactivity of acetaminophen by inhibiting the cytochrome P450 2E1 expression. As an antioxidant flavonoid *in vitro*

baicalin has a strong antioxidant activity toward reactive oxygen species (ROS), including hydroxyl radical (OH \cdot), superoxide anions (O $_2\cdot^-$) and peroxynitrite (ONOO $_2$), and inhibits lipid peroxidation, promoting the repair of DNA single strand breakage caused by H $_2$ O $_2$ in cultured NIH3T3 fibroblasts^[29]. It has long been accepted that Sho-saiko-to functions as a potent anti-hepatic fibrosis agent, and Japanese investigators confirmed that the active components of Sho-saiko-to are baicalin and baicalein of flavonoids, and the chemical structures of baicalin and baicalein are very similar to silybinin, which shows anti-fibrogenic activities^[30,31].

The mechanism of hepatic fibrosis is too complicated for a single drug to resolve. A drug or drug compounds with multi-effect-pathways and multi-effect-targets may have better efficacy than a single drug alone, and could change the current predicament in the therapy of hepatic fibrosis^[19]. Continued progress is essential in order to identify the determinants and dynamics of fibrosis reversibility, to discover additional targets for anti-fibrotic therapy, and to develop customized multi-drug regimens^[32]. Taken together, the results from 2.2.15 cells show that the inhibitory effect of OB combination on HBV antigen secretion and HBV DNA replication is better than that of oxymatrine alone; furthermore, the results from HSC-T6 also show that the suppressing effect of OB combination on α SMA and type I collagen expression is more effective than that of oxymatrine. This study indicates that the addition of baicalin to oxymatrine can strengthen the treatment effect of oxymatrine *in vitro*. However, this finding needs to be verified in *in vivo* studies.

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BASIC RESEARCH

Signaling pathways involved in the inhibition of epidermal growth factor receptor by erlotinib in hepatocellular cancer

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responding to erlotinib treatment could be helpful in predicting the responsiveness of tumors to EGFR-TKIs in the future.

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Key words: Epidermal growth factor receptor; Insulin-like growth factor receptor; Tarceva™; Signal transducer of activation and transcription; Extracellular regulated kinase; Gene expression

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Abstract

AIM: To examine the underlying mechanisms of erlotinib-induced growth inhibition in hepatocellular carcinoma (HCC).

METHODS: Erlotinib-induced alterations in gene expression were evaluated using cDNA array technology; changes in protein expression and/or protein activation due to erlotinib treatment as well as IGF-1-induced EGFR transactivation were investigated using Western blotting.

RESULTS: Erlotinib treatment inhibited the mitogen activated protein (MAP)-kinase pathway and signal transducer of activation and transcription (STAT)-mediated signaling which led to an altered expression of apoptosis and cell cycle regulating genes as demonstrated by cDNA array technology. Overexpression of proapoptotic factors like caspases and gadd5 associated with a down-regulation of antiapoptotic factors like Bcl-2, Bcl-X_L or jun D accounted for erlotinib's potency to induce apoptosis. Downregulation of cell cycle regulators promoting the G₁/S-transition and overexpression of cyclin-dependent kinase inhibitors and gadd5 contributed to the induction of a G₁/G₀-arrest in response to erlotinib. Furthermore, we displayed the transactivation of EGFR-mediated signaling by the IGF-1-receptor and showed erlotinib's inhibitory effects on the receptor-receptor cross talk.

CONCLUSION: Our study sheds light on the understanding of the mechanisms of action of EGFR-TK-inhibition in HCC-cells and thus might facilitate the design of combination therapies that act additively or synergistically. Moreover, our data on the pathways

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common malignancy in the world and is estimated to cause half a million deaths annually. The incidence of HCC is dramatically increasing in the USA, Europe and Asia, due to high prevalence of chronic hepatitis B and hepatitis C virus infections, alcohol disease, diabetes and obesity^[1]. Unfortunately, the majority of patients suffer from advanced disease at presentation. Therefore curative local ablation, surgical resection of HCC, or liver transplantation can be achieved only in a minority of patients. Local tumor destruction, chemoembolization or systemic chemotherapy are the treatment options of advanced HCC. However, overall survival is poor. Apart from chemoembolization, which improves survival in well-selected patients with unresectable HCC, palliative treatment options do not appear to greatly improve overall survival^[2]. Therefore, innovative treatment approaches are urgently needed. Recently, evidence has been accumulated that the epidermal growth factor receptor (EGFR) is a promising target for cancer therapy. A great variety of tumors show abnormal, enhanced and/or constitutive expression of EGFR. Several reports indicate that EGFRs are expressed frequently in human HCC, most likely contributing to the aggressive growth characteristics of these tumors^[3,4]. Especially in poorly differentiated HCCs, EGFR overexpression has been demonstrated to be a negative prognostic factor, since it positively correlated with early

tumor recurrence and the occurrence of extrahepatic metastasis^[3,5]. Hence, the EGFR is a promising target for innovative treatment strategies in HCC.

The EGFR is a member of a family of four closely related receptors: EGFR (ErbB-1), HER-2/*neu* (ErbB-2), HER-3 (ErbB-3), and HER-4 (ErbB-4). Upon ligand binding the EGFR becomes activated by dimerization which leads to subsequent activation of EGFR tyrosine kinase (TK) activity, initiating receptor-mediated signal transduction, cell mitogenesis and cell transformation^[6]. The EGFR downstream intracellular signal transduction pathways include components of Ras/mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase, signal transducer and activator of transcription (STAT), downstream protein kinase C and phospholipase D pathways^[7]. The Ras/MAPK cascade is supposed to be one of the major signaling routes of the EGFR system^[8].

Erlotinib [N-(3-ethynylphenyl)-6,7-bis(2-methoxyethoxy)-4-quinazolinamine] is a novel orally available low-molecular-weight quinazolinamine that acts as a potent and reversible inhibitor of EGFR-TK activity. The mechanism of action of erlotinib is competitive inhibition of the binding of ATP to the TK domain of the receptor, resulting in inhibition of EGFR autophosphorylation^[9]. Single agent activity was observed in pretreated patients with non-small-cell lung cancer (NSCLC), head and neck carcinoma and ovarian cancer^[10]. Recently, the results of the BR.21 phase III trial showed a significant 42.5% improvement in median survival compared to placebo in patients with advanced NSCLC^[11] and the US Food and Drug Administration (FDA) has approved erlotinib for this indication in November 2004.

In a previous study we have shown that EGFR-TK-inhibition by erlotinib potently suppresses the growth of human EGFR-expressing HCC cells by inducing both apoptosis and cell cycle arrest at the G₁/S-transition^[12].

The objective of the current study was to examine the underlying mechanisms of erlotinib-induced growth inhibition in HCC cells. For this purpose we studied the effects of erlotinib on downstream signaling molecules of the EGFR. We used cDNA array technology to investigate the EGFR-TKI-induced modulation of apoptosis- and cell cycle-related genes and Western blot analysis to evaluate changes in the activation of the mitogenic MAPK-kinase- and Jak-STAT-pathways as well as changes in the expression of cell-cycle regulating and antiapoptotic proteins. Additionally, we investigated the influence of IGF-1R-activation on EGFR-mediated signaling and erlotinib's effects on the IGF-1R/EGFR-network.

MATERIALS AND METHODS

Materials

The highly differentiated human hepatocellular carcinoma cell line Huh-7 and the well differentiated hepatoblastoma cell line HepG2 were cultured in RPMI 1640 medium containing 100 mL/L fetal bovine serum and 100 kU/L penicillin and 100 mg/mL streptomycin. Erlotinib hydrochloride was a kind gift from Roche (Penzberg, Germany), cell culture material was from Biochrom (Berlin, Germany); all other chemicals were from Sigma (München,

Germany), if not stated otherwise. Stock solutions were prepared in DMSO and stored at -20°C and were diluted to the final concentration in fresh media before each experiment. In all experiments, the final DMSO concentration did not exceed 5 g/L, thus not affecting cell growth. To evaluate the effects of erlotinib, cells were incubated with either control medium or medium containing rising concentrations of erlotinib.

Drug combination studies

To check for possible additive or synergistic effects, combination treatment of erlotinib plus AG1024 (Calbiochem, Bad Soden, Germany) was studied. The 5 µmol/L or 10 µmol/L of the typhostine AG1024 was combined with 10 µmol/L erlotinib (e.g. approximately its IC₅₀ value). The antineoplastic activities of the combinations were compared to those of each drug alone. For all experiments cell number was evaluated by crystal violet staining as described^[12]. In brief, cells in 96-well plates were fixed with 10 g/L glutaraldehyde, then cells were stained with 1 g/L crystal violet in PBS. The unbound dye was removed by washing with water. Bound crystal violet was solubilized with 2 g/L Triton-X-100 in PBS. Light extinction which increases linearly with the cell number was analyzed at 570 nm using an ELISA-reader.

Western blot analysis

Western blotting was performed as described^[13]. Blots were blocked in 2.5% BSA and then incubated at 4°C overnight with the following antibodies: ERK1/2 (1:500), p-ERK1/2 (1:500), cyclin D1 (1:100), Bcl-X_L (1:200), STAT1 (1:1000), STAT3 (1:1000), STAT5 (1:1000), β-IGF-1R (1:1000), p21^{Waf1/Cip1} (1:200; all from Santa Cruz Biotechnology, CA), p27^{Kip1} (1:2500; Becton-Dickinson, Heidelberg, Germany), p-EGFR, p-STAT1(TYR701), p-STAT3(TYR705), p-STAT5 (TYR694) (all 1:500 and all from Cell Signaling, MA) and p-IGF-1R (1:1500; Biomol, Hamburg, Germany). β-actin (1:5000; Sigma, Deisenhofen, Germany) served as loading control. One representative out of three independent experiments was shown for each Western blot.

RNA extraction and poly(A)⁺ mRNA preparation

Total RNA was extracted from cultured HepG2 cells with RNAClean according to the manufacturer's recommendations (Hybaid, London, UK). Polyadenylated (poly(a)⁺) mRNAs were enriched using magnetic Dynabeads according to the instructions of the supplier (Dyna, Oslo, N). The quality of poly(A)⁺ and total RNA was assessed by agarose gel electrophoresis.

cDNA array

HepG2 cells were treated with 10 µmol/L erlotinib for 48 h to determine erlotinib-induced differential gene expression. Untreated cells served as controls. We used the Atlas Human Apoptosis cDNA array with 205 human cDNAs spotted in duplicate on a nylon membrane (Clontech, Palo Alto, CA) as previously described^[14]. A complete list of the cDNAs and controls as well as their accession numbers is available on the web (http://www.clontech.com/clontech/atlas/genelists/7743-1_HuApop.pdf). The hybridization signals were

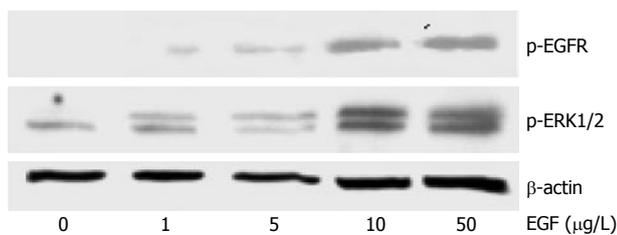


Figure 1 EGF-induced EGFR- and ERK1/2-activation in HCC cells.

photometrically evaluated using TINA software (Raytest Isotopenmessgeräte, Straubenhardt, D). Altered expression of a respective gene is given as a fold increase or decrease compared to the signal of the control. Data analysis was performed as described^[14].

Statistical analysis

The means of three independent experiments of the cDNA expression arrays and the drug combination studies \pm SD are shown. Significance between AG1024 treated samples and AG1024 plus erlotinib treated samples was calculated by Student's two sided *t*-test. $P < 0.05$ was regarded as significant.

RESULTS

EGF-induced EGFR- and ERK1/2-activation

To shed light on the signaling pathways modulated by EGFR-TK inhibition in HCC cells, we investigated the phosphorylation of ERK1/2 known to be involved in EGFR-mediated mitogenic and antiapoptotic signaling. In order to demonstrate the influence of the epidermal growth factor (EGF) on the activation of the EGFR and ERK1/2, serum-starved HepG2 cells were incubated for 15 min with increasing concentrations of EGF (1-50 μ g/L). Serum-starved cells were chosen to exclude the influence of growth factors contained in the fetal calf serum (FCS) of the cell medium. Western blotting of whole cell lysates revealed a dose-dependent increase of activated EGFR and ERK1/2 in response to EGF incubation (Figure 1).

Inhibitory action of erlotinib on EGF-induced EGFR- and ERK1/2 activation

EGF-induced activation of the EGFR and the mitogenic ERK1/2 was blocked by pretreating the cells with erlotinib. HepG2 cells were incubated for 30 min with escalating concentrations of erlotinib (0.1, 1, 10 μ mol/L) and subsequently stimulated with EGF (10 μ g/L). Again, activation of EGFR and ERK1/2 was determined by Western blotting. Erlotinib-untreated cells (control) displayed a pronounced activation of EGFR and ERK1/2 due to EGF-stimulation. Low concentrations of erlotinib (0.1 μ mol/L) completely blocked EGFR-phosphorylation and ERK1/2-activation decreased dose-dependently (Figure 2A).

To mimic *in vivo* conditions, we finally examined the influence of erlotinib (10 μ mol/L) on the activation of ERK1/2 in HepG2 cells stimulated by growth factors contained in the medium's FCS. We revealed a time-dependent decrease in phosphorylation of ERK1/2 (Figure 2B) in HCC cells due to erlotinib treatment (up to 72 h).

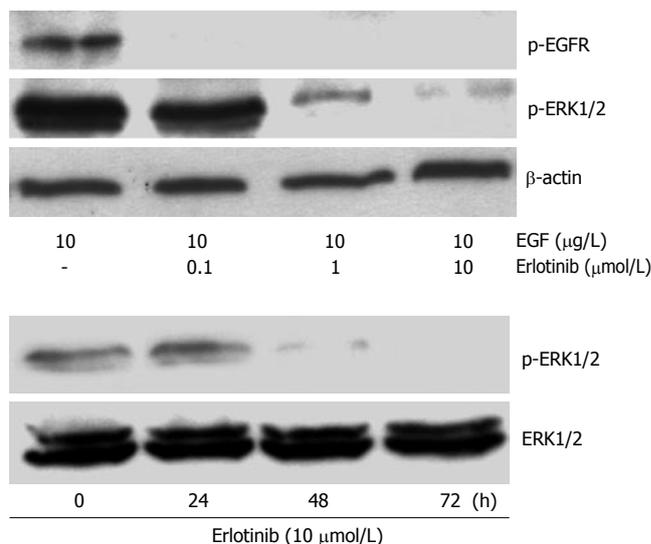


Figure 2 Erlotinib inhibited EGFR (A) and ERK1/2 (B) activation in HCC cells.

STAT expression and EGF-induced activation

EGFR activation is supposed to result in autophosphorylation of Janus-kinases (Jaks) with subsequent activation of mitogenic STATs but has not been investigated in HCC so far. Western blot analyses revealed the expression of STATs 1, 3 and 5 in Huh-7 cells (Figures 3A-C). Moreover, EGF induced STAT activation could be shown for STATs 1 and 3 (Figures 3A and B) but not in the case of STAT5 (Figure 3C). Serum-starved Huh-7 cells were stimulated with EGF (10 or 50 μ g/L, 15 min) and whole cell lysates were used for determinations.

Inhibitory effect of erlotinib on STAT-activation

EGF-induced STAT activation was blocked by pretreating Huh-7 cells with erlotinib. Erlotinib-untreated cells (control) displayed a pronounced activation of STATs 1 and 3 in response to EGF-incubation (10 μ g/L, 15 min). Erlotinib pretreatment (0.1, 1, 10 μ mol/L; 30 min) dose-dependently inhibited the phosphorylation of STATs 1 and 3, whereas the total quantity of STAT 1 and 3 remained unchanged (Figure 4; +: addition of the respective substance, -: absence of the respective substance).

Differential gene expression induced by erlotinib

To further characterize the underlying molecular mechanisms of erlotinib-induced apoptosis and cell cycle arrest, the differential expression of genes related to cell cycle and apoptosis control was investigated using cDNA array technology. HepG2 cells were incubated for 48 h with 10 μ mol/L erlotinib, as a significant arrest of both the cell cycle and apoptosis-induction had been observed under these conditions^[12]. Erlotinib modulated the expression of 25 genes (Table 1). We found an overexpression of genes encoding apoptosis-related cysteine proteases (caspases) 3 and 7, both known to be important enzymes of the apoptotic process. Moreover, growth arrest and DNA-damage inducible (gadd-) genes encoding gadd45 and 153 both associated with induction of apoptosis and growth arrest^[15,16] as well as the insulin-like growth factor

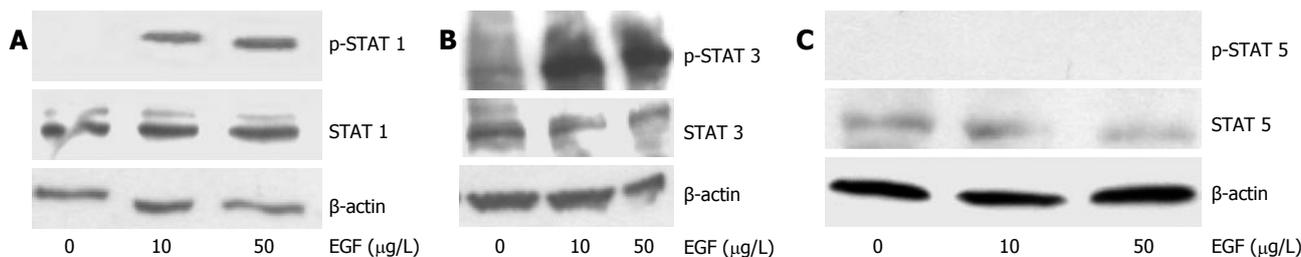


Figure 3 STAT-expression and EGF-induced activation in HCC cells. EGF induced STAT activation could be shown for STATs 1 and 3 (A and B) but not in the case of STAT5 (C).

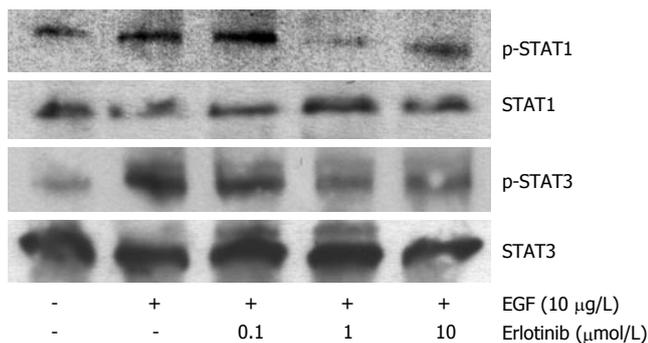


Figure 4 Erlotinib inhibited STAT-activation in HCC cells.

binding protein 6 (IGFBP-6) and cyclin B1, an important molecular regulator of the G₁/S and G₂/M cell cycle transitions^[17] were markedly overexpressed. Genes encoding cell-cycle progression promoting proteins were found to be suppressed (particularly those proteins promoting the transition from the G₁ to the S-phase, e.g. CDK4 or cyclin A2). Additionally, a suppression of anti-apoptotic genes like Bcl-2 or the jun D proto-oncogene was detected as well as of the DNA-replication promoting proliferating cell nuclear antigen (PCNA).

Modulatory effect of erlotinib on expression of cell cycle regulators and antiapoptotic members of the Bcl-2 family

Changes in the expression of important cell cycle and apoptosis regulating proteins due to EGFR-inhibition were assessed by Western blotting. Treating HepG2 cells for up to 48 h with erlotinib (10 μmol/L) resulted in an increase of the cyclin-dependent kinase inhibitors (CDKIs) p21^{Waf1/CIP1} and p27^{Kip1}. The expression of cyclin D1, a protein regulating the transition from the G₁ to the S-phase remained unchanged. Alterations in protein expression of the respective cell cycle regulators occurred within 24 h (Figure 5A). Additionally, a 48-h-treatment of HepG2 cells with 10 μmol/L erlotinib led to a significant suppression of the antiapoptotic Bcl-2 family members Bcl-X_L (Figure 5B) and Bcl-2 (Figure 5C).

Modulation of the MAP-kinase pathway and transactivation of EGFR induced by IGF-1

Comparable to the EGFR, the IGF-1-receptor is associated with carcinogenesis and tumor growth. Thus, we evaluated the mitogenic effects of IGF-1 on Huh-7 and HepG2 cells, both cell lines strongly expressing the IGF-1R^[18]. Besides, we focused on a possible transactivation

Table 1 Transcripts differentially regulated in HepG2 cells in response to erlotinib

GenBankID	Gene name	Mean ¹	SD
M62402	Insulin-like growth factor binding protein 6	5.85	0.73
S66431	Retinoblastoma-binding protein 2	3.22	0.39
U13737	Caspase 3, apoptosis-related cysteine protease	3.18	0.52
Y09392	Tumor necrosis factor receptor superfamily.3.04 member 12 (translocating chain-association membrane protein)	3.04	0.61
M60974	Gadd45	2.37	0.34
S40706	Gadd153	2.31	0.09
M25753	Cyclin B1	2.21	0.47
U37448	Caspase 7, apoptosis-related cysteine protease	2.10	0.34
L16785	Non-metastatic cells 2, protein (NM23B)	2.03	0.22
U23765	BCL2-antagonist/killer 1	0.53	0.13
X51688	Cyclin A2	0.50	0.32
M34065	Cell division cycle 25C	0.49	0.06
L22005	Cell division cycle 34	0.48	0.17
X85134	Retinoblastoma-binding protein 5	0.38	0.12
L25676	Cyclin-dependent kinase 9 (CDC2-related kinase)	0.38	0.05
U66879	BCL2-antagonist of cell death	0.36	0.05
D25216	KIAA0014 gene product	0.35	0.14
X86779	Fas-activated serine/threonine kinase	0.30	0.06
L29220	CDC-like kinase 3	0.28	0.03
X74262	Retinoblastoma-binding protein 4	0.27	0.09
M14505	Cyclin-dependent kinase 4	0.25	0.07
AF010312	LPS-induced TNF-alpha factor	0.24	0.06
U25265	Mitogen-activated protein kinase 5	0.22	0.03
M15796	Proliferating cell nuclear antigen	0.17	0.03
X56681	Jun D proto-oncogene	0.13	0.06

¹ Arithmetic means of ratios (treated:untreated) from three separate array measurements.

of the EGFR in response to IGF-1 treatment. Serum-starved HepG2 cells were treated for 15 min with IGF-1 (50-250 μg/L) followed by the determination of changes in EGFR- and ERK1/2-activation by Western blot. IGF-1 dose-dependently activated the mitogenic MAP-kinase pathway, moreover we could show that IGF-1 treatment in the absence of EGF or other growth factors resulted in an activation of the EGF-receptor, indicating a possible IGF-1R/EGFR cross talk in hepatocellular carcinoma cells (Figure 6).

Inhibitory action of erlotinib on mitogenic effects of EGF and/or IGF-1 and EGFR/IGF-1R receptor cross-talk

Erlotinib-pretreated (30 min, 10 μmol/L) serum-starved

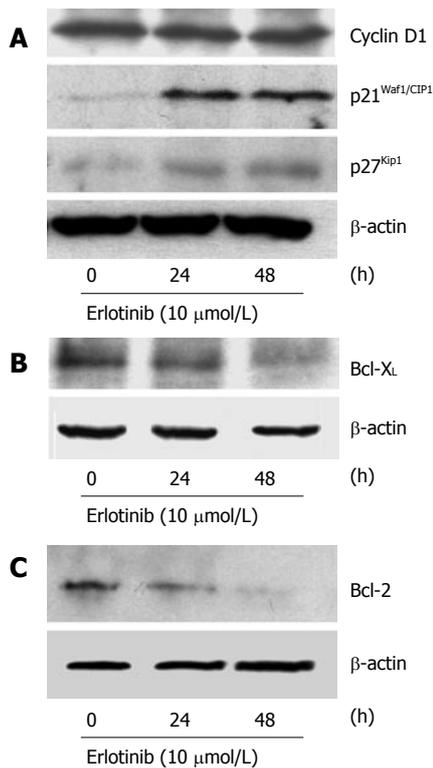


Figure 5 Erlotinib modulated the expression of cell cycle regulators (A) and antiapoptotic members of the Bcl-2 family (B and C).

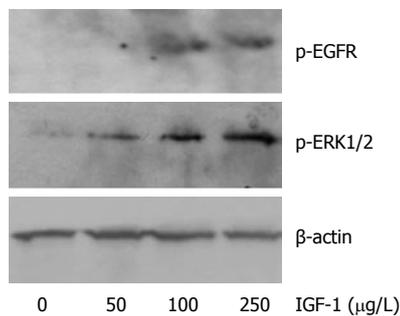


Figure 6 IGF-1-induced activation of ERK1/2 and transactivation of the EGFR.

HepG2 cells were incubated with EGF (10 μg/L) and/or IGF-1 (100 μg/L) for 15 min. Growth factor untreated cells served as control. Comparable to the experiments described above, EGF and/or IGF-1-treatment resulted in the activation of both EGFR and ERK1/2. Erlotinib pretreatment completely inhibited growth factor-induced EGFR activation and reduced p-ERK1/2 levels below control values. Thus, in addition to the blockade of EGF and IGF-1-induced mitogenic signaling, erlotinib potently suppressed IGF-1 induced IGF-1R/EGF-receptor transactivation (Figure 7). In order to exclude unspecific effects of erlotinib on the activation of the IGF-1R by its ligands, we additionally investigated the effects of the EGFR-blocker on the expression and activation of IGF-1R. HepG2 cells were cultured up to 48 h in medium containing 100 mL/L FCS and 10 μmol/L erlotinib. Western blot analysis showed neither changes in the expression of the β-chain of IGF-1R or the IGF-1R

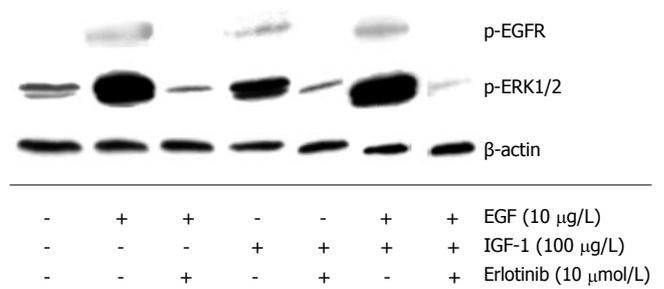


Figure 7 Erlotinib inhibited EGF and/or IGF-1-induced ERK1/2-activation and EGFR/IGF-1R cross talk.

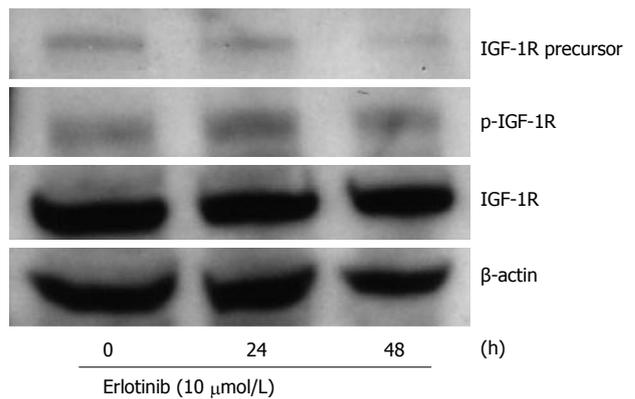


Figure 8 Erlotinib did not influence IGF-1R expression or activation.

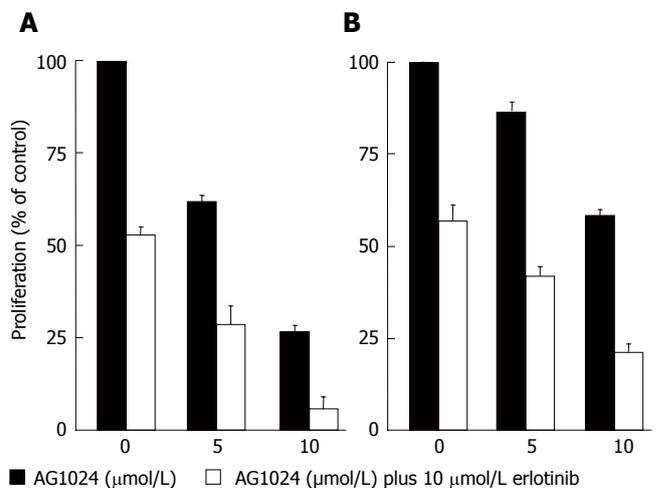


Figure 9 Antiproliferative effects of erlotinib plus AG1024. A: HepG2; B: Huh-7.

precursor nor in the phosphorylation of the receptor as compared to control (Figure 8).

Growth inhibitory effects of simultaneous blockade of EGFR and IGF-1R

On the basis of the results obtained in our investigations on EGFR/IGF-1R receptor signaling, we evaluated the growth inhibitory effects of a simultaneous blockade of the EGFR by erlotinib and the IGF-1R by AG1024. The typhostine AG1024 is a specific inhibitor of the IGF-1R-tyrosine kinase activity. Cells were treated with 5 or 10 μmol/L AG1024 and 10 μmol/L erlotinib for

72 h. Upon treatment with the IGF-1R-TKI alone both HepG2 (Figure 9A) and Huh-7 cells (Figure 9B) displayed a pronounced reduction of cell numbers (Figure 9, black bars). When combining AG1024 with erlotinib, synergistic antineoplastic effects were observed in both cell lines (Figure 9, hatched bars).

DISCUSSION

Treatment options of advanced hepatocellular cancer (HCC) are unsatisfactory, and the prognosis of patients suffering from advanced HCC is poor. New, effective and well-tolerated therapy strategies are urgently needed. The EGF/EGFR system is known to have strong stimulatory effects on the growth of hepatoma cells. Several studies have demonstrated EGFR expression to be a common feature of HCCs^[4,5], underlining the role of the EGFR-TK as a rational target for future hepatocellular cancer treatment. We recently demonstrated that the EGFR-TK-inhibitor erlotinib potently inhibited the growth of EGFR-expressing human hepatocellular cancer cells by a time- and dose-dependent induction of apoptosis and an arrest at the G₁-to-S-transition of the cell cycle^[12]. In accordance, preliminary results of a phase II trial of erlotinib in patients with HCC suggest a clinical benefit by erlotinib^[19]. However, the underlying mechanisms and corresponding molecular events by which erlotinib mediates apoptosis and cell cycle arrest are not yet understood. In this study, we identified signaling molecules involved in erlotinib-mediated apoptosis and cell cycle modulations and showed the functional involvement of the identified pathways.

We demonstrate that EGFR is activated upon EGF binding in HCC cells with a subsequent activation of ERK1/2, a key protein of the mitogen-activated protein kinase (MAPK)-pathway and that erlotinib potently inhibits EGFR activation associated with an inhibition of the mitogenic downstream signaling. MAPKs are important regulators of apoptosis, proliferation and differentiation^[20]. Once activated, ERK1/2 translocates to the nucleus where it acts as a regulator of gene expression of various proteins, e.g. activated ERK1/2 inhibits the expression of the cell cycle inhibitors p21^{Waf1/CIP1}^[21,22] and p27^{Kip1}^[23]. Using Western blotting we could demonstrate an increase of expression of both proteins in response to erlotinib treatment within 24 h. Moreover, ERK1/2-inhibition is known to up-regulate the expression of gadd45^[24] which could be confirmed in this work by cDNA expression arrays. The expression of the gadd45 gene has been correlated with the presence of a strong growth arrest as it interacts with p21^{Waf1/CIP1} to induce cell cycle arrest^[25]. Overexpression of gadd genes causes growth inhibition and/or apoptosis, and combined overexpression of gadd genes leads to synergistic suppression of cell growth^[26]. Furthermore, activated ERK1/2 has been reported to inhibit the apoptotic process by inhibiting caspase activation^[27] and the expression of several antiapoptotic proteins^[20]. In accordance, our previous investigations demonstrated the increase of caspase-3 activity due to EGFR-blockade^[12,28]. In this work cDNA expression arrays revealed that gene expression of caspases 3 and 7 is up-regulated due to erlotinib treatment. In addition to the activation of the proapoptotic caspase

network a decrease of gene and protein expression of antiapoptotic members of the Bcl-2 family as demonstrated by cDNA expression arrays and Western blotting may account for erlotinib's apoptosis inducing capabilities.

Comparable to the MAPK-pathway, Jak-STAT-signaling is involved in cell proliferation and cell cycle progression^[29]. STATs are latent in the cytoplasm and become activated through tyrosine phosphorylation which typically occurs through JAKs or growth factor receptor-TKs. Phosphorylated STATs form homo- or hetero-dimers, enter the nucleus and function as transcription factors. Transcriptional changes concerning apoptosis and cell cycle related genes are similar to those described for ERK1/2^[30]. In normal cells, ligand dependent activation of STATs is a transient process but in tumors the STAT proteins (in particular STATs 1, 3 and 5) are often constitutively activated^[29]. STATs 3 and 5 are noted for the proliferative effects and inhibition of apoptosis whereas the role of STAT1 in oncogenesis and tumor progression is controversial^[31]. Effects of EGFR-TK-inhibition on Jak-STAT-signaling has not been investigated so far. We demonstrated the expression of STATs 1, 3 and 5 in HepG2 cells and the activation of STATs 1 and 3 but not STAT5 due to EGF-stimulation. Erlotinib-treatment inhibited STAT-activation thus contributing to cell cycle arrest and apoptosis-induction.

To shed light on transcriptional changes in response to EGFR-TK-inhibition by erlotinib we performed cDNA expression arrays. As described above, erlotinib increased the expression of genes encoding proapoptotic factors like caspases and gadd whereas the expression of genes encoding antiapoptotic proteins like Bcl-2 or the jun D proto-oncogene was found to be decreased. At the same time we found a different expression of a variety of genes encoding cell cycle regulators: Cell-cycle promoters like CDK4 or cyclin A2 were suppressed, the important molecular regulator of the G₂/M cell cycle transition cyclin B1^[17] was markedly overexpressed probably accounting for a partial G₂/M-block we observed in response to EGFR-blockade in previous investigations^[12,28]. Interestingly, the insulin-like growth factor binding protein 6 (IGFBP-6) was found to be the gene with the strongest overexpression. IGFBPs are a family of six homologous proteins with high binding affinity for IGF-1 and IGF-2. In addition to functioning as simple carrier proteins, IGFBPs in serum regulate the endocrine actions of IGFs by changing the amount of IGF available to activate IGFs, and locally produced IGFBPs act as autocrine/paracrine regulators of IGF action^[32]. Furthermore, recent *in vitro* and *in vivo* findings show that IGFBPs may function independently of the IGFs as growth modulators^[32]. IGFBP-6 differs from the other IGFBPs because it has a markedly higher affinity for IGF-2 than for IGF-1, whereas the other IGFBPs bind the two IGFs with similar affinities^[33]. IGF-2 overexpression is described in several tumor-xenograft models and in human HCCs^[34]. Additionally, a correlation of IGF-2 overexpression with HepG2 and Huh-7 cell growth has been shown^[35] as well as a modulation of IGFBP-expression through the EGFR signaling pathway^[36]. It may be speculated that reduced amounts of bioavailable IGF-2 as a result of EGFR-blockade-induced IGFBP-overexpression contribute to the growth inhibition of hepatocellular can-

cer cells by a further reduction of mitogenic stimuli. However, additional investigations on the complex network of IGF-2R, IGF-2 and IGFBPs have to be undertaken in order to explain our observations and their relevance with anti-EGFR based therapy strategies.

Some of the molecular targets investigated in this study may be used as surrogate biomarkers for anti-EGFR-based therapeutic strategies. The rational selection of cancer patients for EGFR inhibition therapies remains a major challenge because there is no clear correlation between EGFR overexpression and response to EGFR inhibitors^[10]. Thus, the finding of new biomarkers is mandatory. Interestingly, using gadd153 induction as a predictor of clinical response has already been evaluated for paclitaxel treatment of cancer patients^[37].

Like the EGFR, the insulin-like growth factor receptor 1 (IGF-1R) contributes to the growth, survival, adhesion and motility of cancer cells. The IGF-1R is a tetrameric tyrosine kinase receptor which can be activated by either IGF-1 or IGF-2. IGF-1R signaling is mediated through MAPK, phosphatidylinositol-3-kinase (PI3K) and stress-activated protein kinase (SAPK)^[38]. In the present study we showed that the MAPK-pathway was activated by IGF-1 in HCC cells. Moreover, our results revealed an IGF-1R mediated transactivation of the EGFR in HCC cells. Several modes of indirect EGFR activation have been described so far^[39]. As compelling evidence demonstrates the significance of EGFR signal transactivation in human disorders, the components of this signaling mechanism represent promising targets for therapeutic intervention. EGFR transactivation induced by activation of G-protein-coupled receptors, cytokine receptors and voltage-dependent Ca²⁺-channels has been described. Though the exact mechanisms of the receptor-receptor cross talk are not known yet, RTKs are supposed to be important mediators of the transactivation process^[40]. In accordance, our results exhibited erlotinib's potency to inhibit IGF-1 induced transactivation without affecting the activation of the IGF-1R by its ligands. This finding may explain results of previous *in-vitro* studies showing greater antineoplastic activity for EGFR-TK-inhibition in HepG2 and Huh-7 cells^[12,18] than for inhibition of endogenous ligand binding by cetuximab^[28].

In addition to the induction of EGFR-transactivation, IGF-1R is known to be involved in resistance towards anti-EGFR-based therapeutic approaches. This arises from the fact that alternative signaling pathways of the IGF-1R can compensate for a blocked primary EGFR pathway. As IGF-1R is strongly expressed in HCC cells^[34], co-targeting of IGF-1R and EGFR in HCC cells may be a way to avoid or overcome resistance towards EGFR blockade. Combining erlotinib with the IGF-1R-TKI AG1024 resulted in synergistic effects in HepG2 and Huh-7 cells. These results suggest that combination regimens targeting both EGFR and other growth factor receptors such as IGF-1R may simultaneously yield greater anticancer activity than approaches that address only a single receptor and should be investigated more extensively in future studies.

In summary, our data suggest that EGFR-TK-blockade by erlotinib leads to an inhibition of the mitogenic MAPK pathway as well as to an interruption of STAT-

mediated signaling, resulting in a different expression of apoptosis and cell cycle regulating genes in HCC cells. Overexpression of proapoptotic factors like caspases and gadd5 associated with a down-regulation of antiapoptotic factors like Bcl-2, Bcl-X_L or jun D may account for erlotinib's action to induce apoptosis. Downregulation of cell cycle regulators promoting the G₁-to-S-transition and overexpression of cyclin-dependent kinase inhibitors and gadd5 contribute to the induction of a G₁/G₀-arrest in response to EGFR-TK-inhibition by erlotinib. Moreover, our results point at the transactivation of EGFR-mediated signaling by the IGF-1R and show erlotinib's inhibitory effects on the receptor-receptor cross talk mechanisms. Finally we demonstrate that synchronous targeting of EGFR and IGF-1R yields greater antineoplastic effects than approaches that address only a single receptor. To conclude, our study sheds light on the understanding of the mechanisms of action of EGFR-TK-inhibition in HCC-cells and thus might facilitate the finding of combination therapies that act additively or synergistically. Moreover, our data on the pathways activated by erlotinib could be helpful in predicting the responsiveness of tumors to EGFR-TKIs in the future.

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BASIC RESEARCH

5-Fluorouracil-related enhancement of adenoviral infection is Coxsackievirus-adenovirus receptor independent and associated with morphological changes in lipid membranes

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Abstract

AIM: To evaluate the mechanism underlying the effects of 5-Fluorouracil (5-FU) on adenoviral infection.

METHODS: Low and high Coxsackievirus-Adenovirus Receptor (CAR) expressing human colon carcinoma cell lines were treated with 5-FU and two E1-deleted adenoviral constructs, one transferring GFP (Ad/CMV-GFP) the other bax (Ad/CEA-bax). The number of infected cells were monitored by GFP expression. To evaluate the effects of 5-FU in a receptor free system, Ad/GFP were encapsulated in liposomes and treated with 5-FU. Ad/GFP release was estimated with PCR and infection of 293 cells with the supernatant. Electron microscopy of the Ad5-GFP-liposome complex was made to investigate morphological changes of the liposomes after 5-FU.

RESULTS: Infection rates of all cell lines increased from 50% to 98% with emerging 5-FU concentrations. The enhanced viral uptake was independent of the CAR expression. Additionally, 5-FU treated liposomes released 2-2.5 times more adenoviruses. Furthermore, 5-FU-treated liposomes appeared irregular and porous-like.

CONCLUSION: adenoviral uptake is enhanced in the presence of 5-FU irrespective of CAR and is associated with morphological changes in membranes making the combination of both a promising option in gene therapy.

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Key words: 5-Fluorouracil; Coxsackievirus-adenovirus receptor; Adenoviral infection; Gene therapy

INTRODUCTION

It is widely accepted that cancer is the endpoint of an accumulation of genetic mutations that result in a cellular phenotype characterized by uncontrolled growth and reduced apoptosis. Consequently, therapeutic strategies, which address the genetic lesions and thus kill cancer cells, are reasonable. This concept has made virus-mediated gene therapy an ideal candidate for therapeutic approaches either alternative or complementary to chemotherapy or radiotherapy. Human adenoviruses are widely used as delivery systems but adenovirus (Ad)³ uptake is dependent on expression of Coxsackievirus-adenovirus receptor (CAR). Unfortunately, tumor cells are usually characterized by a reduced expression of CAR that binds the fiber knob domain of the Ad serotypes 2 and 5^[1]. Cells lacking this receptor are more resistant to adenoviral infection and, consequently, they are poor targets for Ad-associated tumor therapies^[2-5]. Further, it has been shown that the treatment of colorectal cancer with the replication-selective Ad *d*/1520 in combination with 5-Fluorouracil (5-FU) was more efficient in inducing apoptosis than the administration of the two agents separately^[6-9]. Therefore we evaluated the mechanism underlying the effects of 5-FU in the context of adenoviral infection in this study. To better understand the role of 5-FU in adenoviral infection of tumor cells, we used two E1- and replication-deficient adenoviral mutants expressing GFP (Ad-GFP) to infect colorectal cancer cell lines that show different CAR expression. A significantly higher number of GFP-expressing cells were observed after treatment with 5-FU and Ad-GFP compared to Ad-GFP alone. The effect of 5-FU was even more striking in a cell line with low CAR expression (SW480) indicating that a CAR-independent mechanism may be responsible for the transport of Ad through the cell membrane. This enhancement

of infection was dose-dependent and maximal with simultaneous application of 5-FU and Ad-GFP. To assess this effect in a CAR-independent pathway, Ad-GFP was encapsulated in liposomes, which were treated with 5-FU. Supernatants of these liposomes contained 2.4 times more Ad-GFP after 5-FU treatment compared to controls. In addition, morphological changes in the lipid membranes were seen by electron microscopy. In conclusion, we could demonstrate that simultaneous treatment with 5-FU enhances adenoviral uptake into tumor cells. More importantly, this effect could be observed irrespective of CAR expression and 5-FU favors the crossing of the viral protein capsid through the lipid membranes. Regardless of the underlying mechanism, the combination of adenoviral and 5-FU treatment might be of significant importance in the context of gene transfer.

MATERIALS AND METHODS

Cell lines and culture conditions

All cell lines used in this study were purchased from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and were grown in the appropriate media containing 100 mL/L FCS, 10 g/L Pen/Strep (PS) and 10 g/L glutamine. The human embryonic kidney 293 cells were grown in DMEM, the human colon adenocarcinoma cells DLD-1, LOVO, SW480 and SW620 in RPMI 1640, and the RKO cells in McCoy.

Recombinant virus construction and purification

The adenoviral vectors for gene expression were constructed with the AdEasy system^[10]. Briefly, Ad-CEA_{abax} was constructed with the pAdTrack vector containing the CEA promoter in front of the bax gene and the gene for GFP under the control of the CMV promoter, whereas Ad-GFP was constructed with the pAdTrack vector containing GFP under the control of the CMV promoter. The resulting plasmids were then transformed into *Escherichia coli* cells with pAdEasy-1. The recombinant adenoviruses were generated in the 293 cells (E1-transformed) and purified by CsCl gradient ultracentrifugation^[11]. The titer was determined by counting the green cells after 48 h.

Cell culture and infection

For Ad infection of SW480 the cells were seeded onto 96-well plates at 1500 cells/well and grown for 2 d. For the 5-FU pretreatment, the cells were first incubated at 37°C in RPMI/FCS/PS containing 5-FU at concentrations of 2, 10, 30 and 50 µmol/L. After 2 h the medium was removed, the cells were washed and infected with Ad-GFP at 300 pfu/well in 200 µL medium without the drug. For the co-treatment, the cells were infected with Ad-GFP diluted in 200 µL medium containing 5-FU at the concentrations reported above. Controls were incubated with medium without 5-FU. The number of green cells was counted after 24, 48 and 72 h with a fluorescence microscope for GFP expression. For the infection of the Lovo and SW480 cells with Ad-CEA_{abax}, the cells were plated onto 60 mm dishes at a density of 2×10^5 cells/

dish. After 1 d, the cells were infected with the virus (1 MOI) in 1 mL medium without FCS and PS. After 30 min incubation at 37°C, the cells were treated with the medium containing FCS, PS and 5-FU at the final concentrations of 2 µmol/L for Lovo and 20 µmol/L for SW480. For the treatment of the colon cancer cells with Ad-GFP and 5-FU, 5-BrU, 5-FC, DOC, or taxol, the cells were seeded onto 6-well plates at 2×10^5 cells/well. After 1 d, Ad-GFP was added at 3×10^4 pfu/well in 1 mL medium without FCS and PS. After 30 min incubation at 37°C, 2 mL growth medium were added containing FCS, PS and the drug at the following final concentration: 4 µmol/L for 5-FU, 5-BrU, 5-FC and DOC, and 5 µg/L for taxol. The number of green cells was counted 48 h after infection.

Liposome preparation

To assess the effects of 5-FU of lipid membranes and the ability of Ad to penetrate the membranes, we encapsulated Ad in liposomes with and without 5-FU. The release of Ad was measured by PCR for E4orf6. In addition, the number of infective particles was evaluated by infection of 293 cells with the supernatant. The liposomal formulation of Ad was prepared as follows: Ad-GFP (3×10^4 pfu) was mixed with lipofectamine (Invitrogen) that was brought to a final concentration of 0.4 g/L with PBS. After 2 h incubation at room temperature, 5-FU was added to the final concentration of 20 µmol/L. The mixture was incubated overnight at room temperature and then centrifuged at 2060 g for 50 min at 20°C. The supernatant (100 µL/well) was diluted to 1 mL with DMEM without FCS and PS and added to the cells that were then incubated for 30 min at 37°C. Afterwards, 2.5 mL DMEM containing FCS and PS were added and the cells were further incubated. For the experiment with the liposome-encapsulated Ad-GFP, the 293 cells were plated into 6-well plates at 2×10^5 cells/well and were grown overnight.

PCR analysis

The following adenoviral DNA samples (each 2 µg) were prepared for PCR: DNA alone, DNA in the presence of 9.6 µmol/L 5-FU, DNA/lipofectamine (0.2 g/L), DNA/lipofectamine (0.2 g/L) in the presence of 9.6 µmol/L 5-FU. The samples were incubated overnight at room temperature, then centrifuged at 2060 g for 35 min at 18°C. For control, an additional sample of DNA/lipofectamine (0.2 g/L) was prepared and treated with a lysis buffer for 1 h at 4°C prior to centrifugation. The DNA was precipitated from the supernatants upon addition of NaOAc and isopropanol, isolated by centrifugation, washed with 750 mL/L ethanol and dried. The PCR was performed by using the Taq PCR Master Mix (Qiagen). The resultant PCR products were then resolved on a 1.5% agarose gel containing 0.25 mg/L of ethidium bromide.

Flow cytometric analysis

Single cell suspensions were fixed in 700 mL/L ethanol and incubated with 50 g/L PI and 20 g/L RNase for 15 min. at 37°C. Flow analysis was done at 488 nm excitation and > 525 nm Em range collected for GFP fluorescence. Elite Software 4.0 (Coulter Corp, Miami, FL) and Multi

Cycle DNA Analysis program software (Phoenix Flow Systems, San Diego, CA).

Western blot analyses

Protein concentration was determined by using the BC assay (Interchim, Montluçon, France). The protein samples [40 µg for the CAR detection, 60 µg for the bax detection and 50 µg for the fiber detection (samples were not cooked)] were separated on SDS-polyacrylamide gels (100 g/L for the CAR detection, 150 g/L for the bax detection and 75 g/L for the fiber detection), and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). The blots were blocked with 50 mL/L nonfat dry milk in 1 g/L Tween 20 PBS (TPBS-MLK) for 1 h at room temperature, then incubated overnight at 4°C with the appropriated antibodies: CAR (goat polyclonal antibody, Santa Cruz) at the dilution of 1:285, bax (polyclonal rabbit anti-human, PharMingen) at the dilution of 1:1000, fiber (polyclonal mouse, NeoMarkers, Fremont, CA) at the dilution of 1:1000 and actin (goat polyclonal antibody, Santa Cruz) at the dilution of 1:1000. After washing with TPBS, the following secondary antibodies were added at the dilution of 1:500 and incubated for 1 h at room temperature: HRP-conjugated IgG from donkey anti-goat for CAR and actin detection, and from goat anti-rabbit for bax detection. The proteins were finally visualized by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).

CAR blockade

SW480 cells were plated into 6 well plates at 2×10^5 cells/well and incubated with CAR antibody (goat polyclonal antibody, Santa Cruz) at the dilution of 1:1000, 1:750, 1:500, 1:250, 1:100 and 1:50 for 24 h. The cells were incubated with Ad-GFP and the number of green cells was counted 48 h after infection.

Transmission electron microscopy

The following solutions were analyzed by electron microscopy (EM): (A) lipofectamine at 0.5 g/L, (B) lipofectamine at 0.5 g/L treated overnight with 20 µmol/L 5-FU, (C) Ad-GFP (4×10^8 pfu/L) complexed with lipofectamine at 0.5 g/L, (D) Ad-GFP (4×10^5 pfu/mL) complexed with lipofectamine at 0.5 g/L and incubated for 1 h at room temperature prior to treatment overnight with 20 µmol/L 5-FU. The EM samples were prepared by using the negative stain procedure. Briefly, a drop of each sample was deposited on a copper grid and coated by a formvar/carbon film. The film was then stained with 20 g/L tungsten phosphoric acid and dried on air. The EM images were recorded on a Zeiss instrument operating at 80 kV.

RESULTS

5-FU augments the viral infection rate of both high and low CAR-expressing cell lines

Several cell lines (DLD-1, Lovo, SW480 and SW620) were treated with Ad-GFP or Ad-CEAbax at a MOI that infected 50% of the cells. The infection of all cell lines was surprisingly efficient (up to 98%) when the adenoviral constructs were used in combination with 5-FU, as

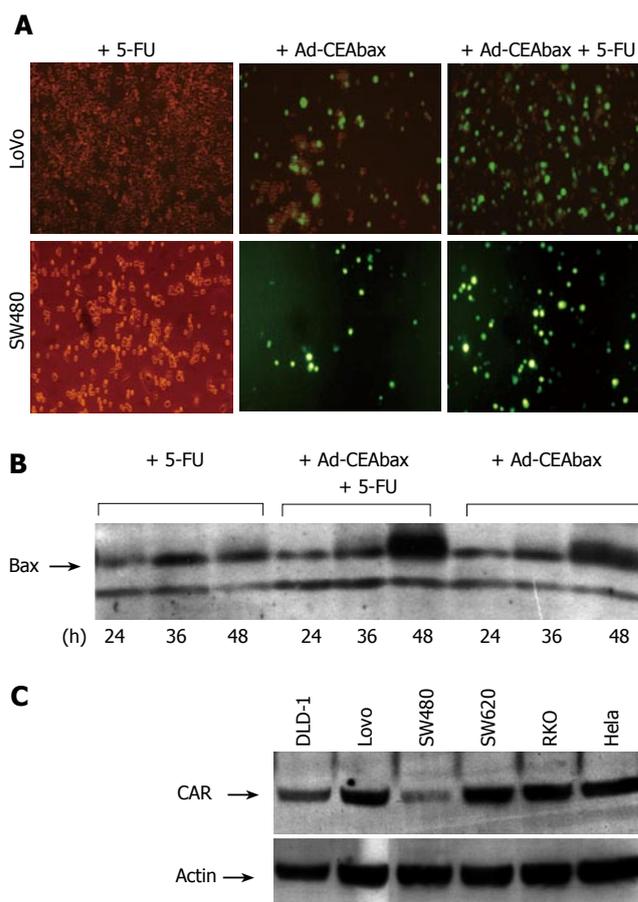


Figure 1 Effect of 5-FU on the adenoviral infection of colon carcinoma cell lines differing in CAR expression. The drug was used at the dose of 2 µmol/L for the Lovo cells and of 20 µmol/L for the SW480 cells. The virus (Ad-CEAbax) was used at 1 MOI. The cells were treated with 5-FU alone, or infected with the virus in the absence and in the presence of 5-FU (A). The expression of bax with 5-FU and/or the virus was controlled by western blot after 24 h, 36 h and 48 h incubation (B). The expression level of CAR on the colon carcinoma cells line was confirmed by western blot analysis (the expression of actin is reported as a control for loading) (C).

indicated by the high population of green cells in Lovo and SW480 cell examples (Figure 1A and 2A). In accordance with GFP expression, an increased expression of bax was detected (Figure 1B) after Ad-CEAbax infection and 5-FU. The RKO cells died after simultaneous treatment and thus the number of green cells decreased after 24 h (Figure 4). As control 293 cells were transfected with the CMV Promoter/GFP DNA and treated with 10 µmol/L 5-FU. No difference in the amount of green cells was seen after 5-FU indicating that 5-FU does not interfere with the transcription of the reporter gene (data not shown). In addition, SW480 cell lysates of 5-FU treated (10 µmol/L) and control cells were blotted for adenoviral fiber protein after treatment with two different adenoviral MOI (200 and 50). In both cases more fiber protein could be detected in 5-FU treated cells (Figure 2B) indicating a higher amount of intracellular adenovirus. 5-FU enhanced the uptake of Ad not only in cell systems expressing CAR at high levels, such as the Lovo cells, but even more in cells with a low-level CAR expression (Figure 1C), indicating that the effect might not be dependent on CAR expression. To block the function of CAR SW480 cells were incubated with

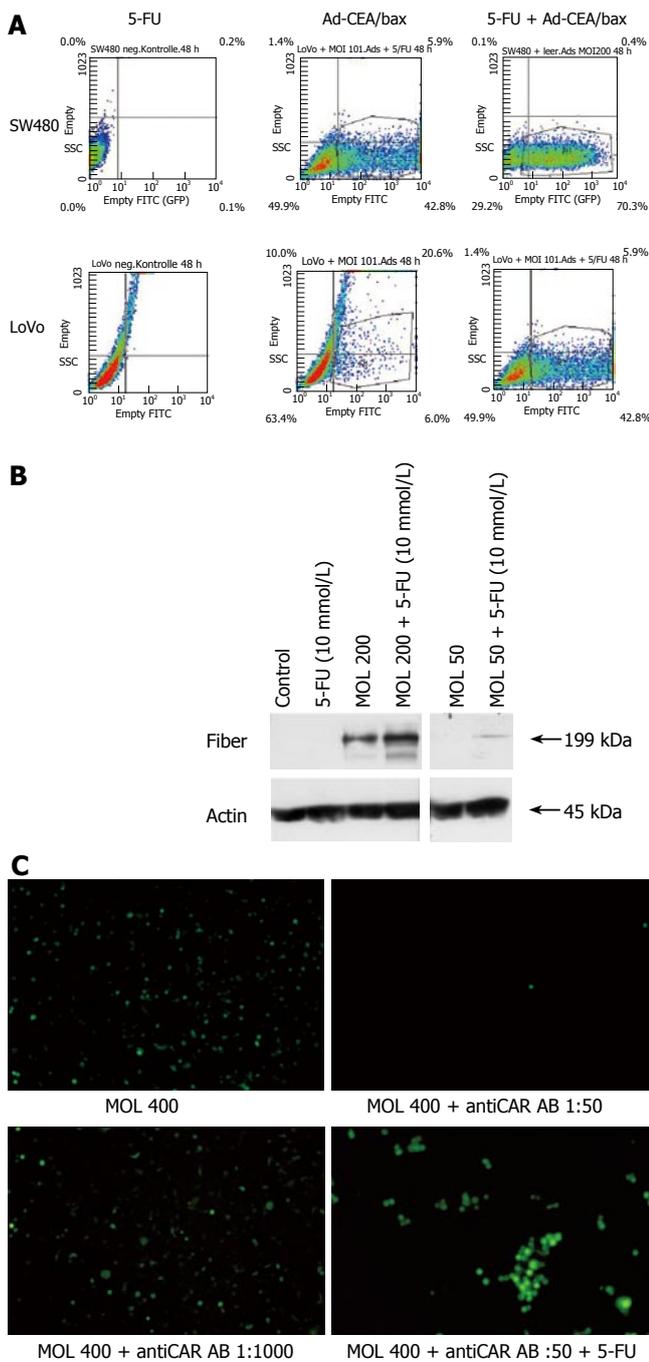


Figure 2 The number of GFP expression cells was measured by flow cytometry in SW480 and LoVo cells (A). Treatment with 5-FU resulted in increased number of GFP expression cells and increased intensity of GFP expression in both cell lines. Intracellular adenoviral fiber protein was assessed by western blot analysis of SW480 cell lysates (B) after Ad-GFP treatment. In control cells and 5-FU treated cells no fiber protein could be detected. Low amounts of fiber protein could be seen in Ad-GFP treated cells dependent on Ad-GFP concentrations (MOI of 200 and 50). Additional 5-FU treatment increased fiber protein in whole cell lysates indicating enhanced adenoviral uptake. Function of CAR was blocked by anti CAR antibody at a concentration of 1:500 but 5-FU enhanced the number of GFP expressing cells even at anti CAR antibody concentrations of 1:50 (C).

antibody against CAR (Santa Cruz, N-17) in increasing concentrations from 1:1000 to 1:50 for 24 h and incubated with Ad (MOI 400). Viral uptake was blocked at higher concentrations than 1:500 but treatment with 5-FU still enhanced viral uptake (Figure 2C) accounting for a CAR independent mechanism of 5-FU.

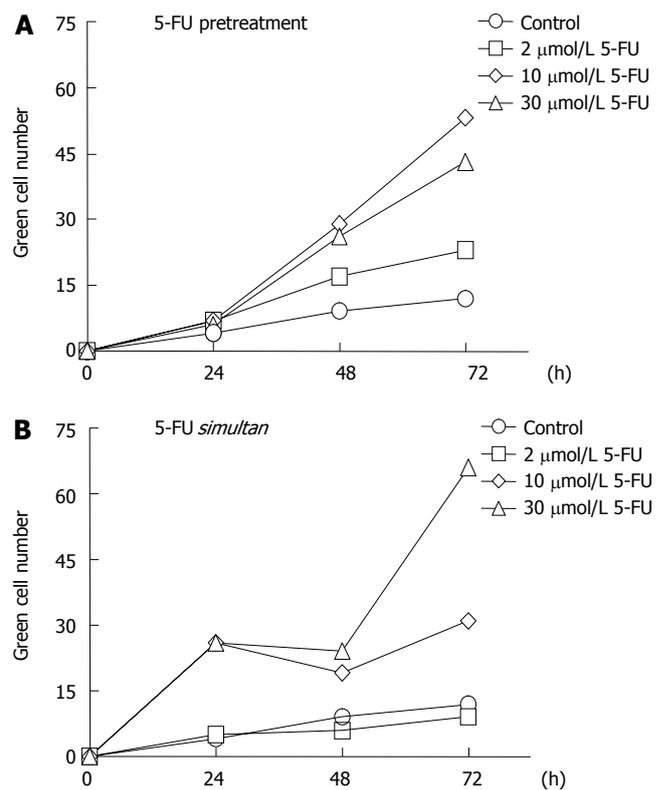


Figure 3 Dependence of adenoviral efficacy on the sequencing of 5-FU and virus administration to the SW480 cells. Two h postincubation with the drug at the indicated concentrations, the drug was removed and the cells were infected with the adenoviral construct Ad-GFP (A). Alternatively, the cells were infected with the virus in combination with 5-FU (B). The number of green cells was counted after 24, 48 and 72 h infection. The reported data points are the average of three experiments and the standard deviation values were in the range of 10%-15% (the error bars were not reported for clarity).

Efficacy of 5-FU on Ad uptake is dependent on drug concentration

The GFP expression in 5-FU-pretreated SW480 cells was significantly higher than in untreated cells, and the number of cells producing the fluorescent protein increased with increasing concentrations of the anticancer agent up to 10 μmol/L. Indeed, after 48 and 72 h the rate of Ad infection in cell pretreated with 10 μmol/L 5-FU was more than twice the infection rate obtained with 2 μmol/L 5-FU (Figure 3A). At the higher concentrations of 30 μmol/L, however, the drug was shown to be moderately less effective, a phenomenon that could be due to an inhibitory effect of 5-FU on the viral DNA replication (data not shown). All experiments were done in triplicate and the standard deviation was less than 15%. A superior efficacy of 5-FU was observed by its simultaneous application with Ad, which led to an improvement not only in the yield but also in the rate of the infection, especially when the drug was used at 30 μmol/L. Under these conditions the number of green cells counted after 24 h was three times higher than in the case of the drug pretreatment and there was an increment of more than 50% in the density of green cells after 72 h (Figure 3B).

The increase in cellular Ad uptake is a specific response to 5-FU

The positive effect of 5-FU on Ad infection could be

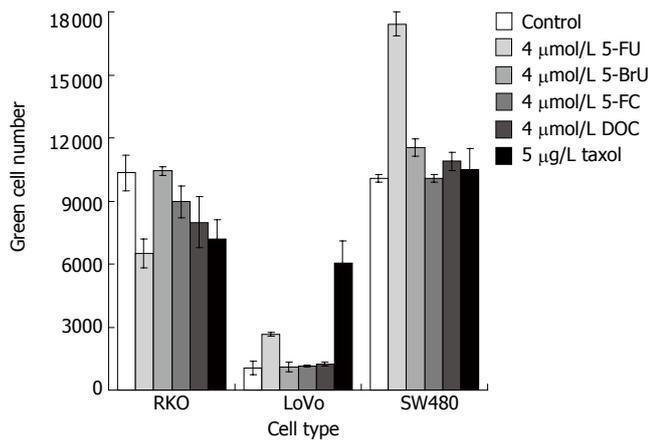


Figure 4 Other pyrimidine-based drugs do not show the positive effect of 5-FU on adenoviral infection. The colon carcinoma cell lines were treated concurrently with the virus Ad-GFP and 5-FU, 5-BrU, 5-FC, DOC or taxol, each at the indicated concentration. The number of the infected green cells was counted after 40 h infection. The reported data are the average of three experiments and the error bars indicate the standard deviation values.

related to its function as a chemotherapeutic agent or to its structural and chemical characteristics. To assess which structural and chemical features of 5-FU might play a role in the entry of Ad into the cells, the two compounds 5-BrU and 5-FC, which are pyrimidine derivatives as 5-FU, were used in combination with Ad. In 5-BrU the halogen is bigger and less electronegative than in 5-FU. In 5-FC an amino group is present at position 4 instead of a carbonyl group as in 5-FU, thus conferring a higher hydrophilicity to the molecule. In both cases, the level of the infection did not change with respect to Ad alone, indicating that the ability of 5-FU to favor Ad infection is specific and not common to other molecules structurally-related to 5-FU. Additionally to other chemotherapy drugs, such as taxol^[12], the cell membrane-destabilizing bile salt sodium deoxycholate^[13] was used in combination with Ad. In the case of the DLD-1 and SW480 cells only 5-FU positively influenced the cellular entry of Ad, whereas taxol did not show any effect. In contrast, the infection of the Lovo cells was improved by both anticancer drugs, with taxol being twice more effective than 5-FU (Figure 4). The effect of these drugs was only moderate in the RKO cells, which do express high levels of CAR.

5-FU increases the release of Ad from liposomal formulations

Based on the observation that 5-FU positively affected the Ad uptake independently of CAR, we postulated a 5-FU-mediated transfer of Ad through lipid membranes. To investigate whether 5-FU exerts any effect on ordered lipid structures, a liposome mixture of DOSPA/DOPE at a 3:1 ratio was used to encapsulate the Ad, and the resulting complex was then treated with 5-FU overnight. After centrifugation, the number of infective particles in the supernatant was tested on 293 cells, which provide a system for the replication of E1B deleted Ad mutants. As shown in Figure 5A, the green cell number obtained from the infection with the supernatant of the 5-FU-treated Ad-liposome preparation was at least 2.4 times higher than that obtained from the infection with the supernatant of

the same preparation but without 5-FU. This is indicative of an augmented viral concentration in the supernatant as the result of the incubation of the liposome-entrapped Ad with 5-FU. In order to control whether the treatment with 5-FU could induce the release of adenoviral DNA from the liposomes, samples of DNA-liposome solutions with and without 5-FU were subjected to PCR and then analyzed by agarose gel electrophoresis. No adenoviral DNA was visualized by ethidium bromide staining, indicating that there was no release of the DNA component from the liposome complex in the presence of 5-FU. As a control, the DNA-liposome complex was subjected to liposome disruption prior to PCR and the resultant PCR product was visualized on the agarose gel, as it was expected (Figure 5B).

5-FU induces a morphological change of the lipid layers

The EM images of the liposomal formulations upon 5-FU treatment showed a multilayer motif that was characterized by an irregular thickness of the liposome as a result of the disappearance of an ordered layer structure in some regions (Figure 5C). Similarly, in the case of the Ad-liposome complex treated with 5-FU, the lipid surface was not uniform, but showed some bright spots that are probably indicative of perturbations in the packing and ordering of the multilayers.

DISCUSSION

In this study we could demonstrate that 5-FU increases the effectiveness of adenoviral uptake into colorectal cancer cells, thus overcoming the resistance of several colorectal cancer cells to adenoviral treatment. This effect is independent of CAR expression on the cell surface and could be confirmed in a receptor free system. In addition this effect is associated with changes in lipid membranes. Thus, the combination of the anti-tumor drug 5-FU with adenovirus enhances gene transfer capabilities of the virus in colon cancer cells. The synergistic effect of oncolytic Ad (*d11520*) and chemotherapeutic agents, such as 5-FU and cisplatin^[6-9], is already known, but the reason for such behavior is not completely elucidated. One proposed explanation is the enhancement of cell chemosensitivity induced by viral replication, probably through the expression of the E1A gene that occurs after Ad infection and increases tumor cell killing^[14]. Nevertheless, this mechanism does not apply to all cell systems, because it can occur only in those cells that are not resistant to the Ad entry and, more strikingly, our data demonstrate that even E1-deleted constructs, which are replication-deficient, are more effective in the context of 5-FU treatment.

As stated before, a replication defect of human Ad was used to transfer CMV promoter/GFP DNA but 5-FU could probably increase transcription of the CMV promoter similar to FDXR induction by p53 in cells treated with 5-FU^[15]. Therefore we transfected cells with CMV promoter/GFP plasmid and could not see any enhancement of GFP expression with 5-FU. In addition, higher amounts of adenoviral fiber protein were detected in cell lysates after Ad and 5-FU treatment indicating more Ad particles within the cells

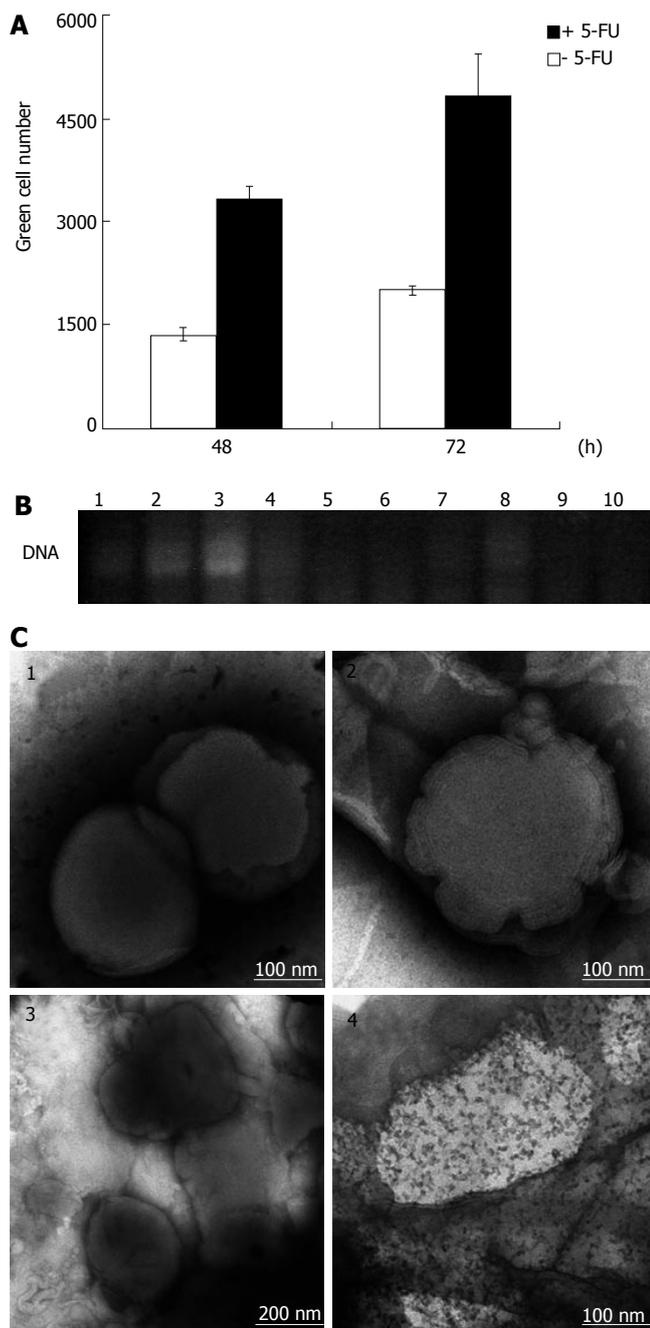


Figure 5 Increased release of viral particles from liposome-encapsulated adenoviral formulations. 293 cells were infected with the supernatant from centrifuged liposome-entrapped Ad-GFP solutions with and without 5-FU incubation. The number of the infected green cells was counted after 48 h and 72 h infection. The reported data are the average of three experiments and the error bars indicate the standard deviation values (**A**). On the other hand, the 5-FU treatment of liposome-encapsulated viral DNA did not lead to any DNA release, as confirmed by PCR analysis. Following samples (each loaded in duplicate) were separated by agarose gel electrophoresis: DNA alone (lanes 1 and 2), DNA and 9.6 $\mu\text{mol/L}$ 5-FU (lanes 3 and 4), DNA and lipofectamine (lanes 5 and 6), DNA with lipofectamine and lysis buffer (lanes 7 and 8), DNA with lipofectamine and 5-FU 9.6 $\mu\text{mol/L}$ (lanes 9 and 10) (**B**). EM images of liposomal preparations. (1) lipofectamine, (2) lipofectamine after treatment overnight with 5-FU, (3) Ad-GFP complexed with lipofectamine, (4) Ad-GFP complexed with lipofectamine after treatment overnight with 5-FU (**C**).

and suggesting that the higher amount of green cells is a result of higher Ad uptake into cells. Furthermore, it was proposed that the loss of sensitivity of cancer cells to Ad treatment is generally a consequence of loss of CAR

representing a severe limitation of the application of gene therapeutic strategies with Ad as a gene delivery system^[11]. Interestingly, although the binding of Ad to its receptor is suggested to be the first step for infection, our results indicated that the presence of CAR does not provide a guarantee for efficient viral infection. Indeed, not only cells expressing low levels of CAR, such as the SW480 cells, but also cells expressing normal levels of CAR, such as the DLD-1, Lovo and SW620 cells, were found to be Ad-resistant. However, simultaneous treatment with 5-FU and Ad could enhance the sensitivity to adenoviral infection in all tested cell systems but the effects of 5-FU treatment was more impressing in cell lines which are difficult to infect with Ad. To assess the effect of 5-FU we blocked CAR and could still see enhanced viral uptake. Interestingly, the positive effect of 5-FU was superior when both Ad and 5-FU were added simultaneously in comparison to the pre-incubation of the cells with the drug before the addition of Ad. This suggests that the entry of Ad into cells may occur independently of the production or degradation of effectors caused by 5-FU treatment. Therefore we assessed the capability of Ad to pass through liposomal membranes in the presence of 5-FU and, in accordance with this hypothesis, we observed an increased adenoviral release from liposomes treated with 5-FU.

In light of our experiments on cells differing in CAR expression, we postulate that Ad uptake could be based on a mechanism alternative to that requiring the binding to the Ad receptor. In the presence of CAR, Ad is delivered into the cell via an internalization process involving receptor-mediated endocytosis^[16]. Alternatively, in the absence of CAR, the adenoviral protein capsid is likely to interact directly with the phospholipid layers of the cell membrane; however, such interaction results in an effective intracellular transfer only in the presence of 5-FU. The intra- and extra-cellular drug diffusion across the membrane could be coupled to temporary changes in the packing and ordering of the lipid bilayers building the cell membrane, which, in turn, could become more accessible to external agents. Nevertheless, the potential effects of 5-FU on the membrane must be different from those of other amphiphilic molecules that are known to exert a lytic action on membranes, such as DOC^[17], as suggested by the observation that this bile salt did not increase the Ad uptake. Previous studies have reported on morphological changes of cells after treatment with adenoviruses^[18] or with detergents^[17]. Moreover, the interactions between biological or model membranes and hydrophobic drugs, such as 1, 4-dihydropyridines^[19] and benzocaine^[20], have been investigated in detail, but the exact mechanism of 5-FU with cell membranes remains elusive. The human colon cancer cells tested in this work generally became bigger and adopted a spindle shape upon incubation with 5-FU. This supports our hypothesis that, beside its well-known anti-cancer action, 5-FU may exert a potential disturbing effect at the cell membrane. Interestingly, 5-FU changes intestinal absorption in rat of dextran^[21]. However, the exact mechanism of adenoviral passage through lipid membrane in the presence of 5-FU remains unclear.

In conclusion, we suggest that 5-FU might play a role

in increasing the sensitivity of cells for environmental influences by changes in the phospholipid bilayers of cell membranes in addition to its chemotherapeutic property. This would be especially useful for the transport of therapeutic compounds independently of the presence or absence of specific cell surface receptors, as in the case of the transfer of Ad into CAR-negative cells. On the other hand, side effects of adenoviral infections during high-dose chemotherapy might not only be based on a suppressed immune system, but, if 5-FU enhances viral uptake, flue-like side effects of the 5-FU chemotherapy might be caused by a higher adenoviral uptake into cells.

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Involvement of P53 and Bax/Bad triggering apoptosis in thioacetamide-induced hepatic epithelial cells

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Abstract

AIM: Thioacetamide (TAA) has been used in studying liver fibrosis and cirrhosis, however, the mechanisms of TAA-induced apoptosis in liver are still unclear. The hepatic epithelial cell line clone 9 was cultured and treated with TAA to investigate the causes of cell death.

METHODS: The cell viability of TAA-induced clone 9 cells was determined using MTT assay. Total cellular GSH in TAA-induced clone 9 cells was measured using a slight modification of the Tietze assay. The activity of caspase 3 in TAA-induced clone 9 cells was monitored by the cleavage of DEVD-p-nitroaniline. TUNEL assay and flow cytometry were applied for the determination of DNA fragmentation and the proportion of apoptosis in TAA-induced clone 9 cells, respectively. The alterations of caspase 3, Bad, Bax and Phospho-P53 contents in TAA-induced clone 9 cells were measured by Western blot.

RESULTS: The experimental data indicated that TAA caused rat hepatic epithelial cell line clone 9 cell death in a dose- and time-dependent manner; 60% of the cells died (MTT assay) within 24 h after 100 mg/L TAA was applied. Apoptotic cell percentage (TUNEL assay) and caspase 3 activities were highest after 100 mg/L TAA was added for 8 h. The release of GSH and the elevation in caspase content after TAA treatment resulted in clone 9 cell apoptosis *via* oxidative stress and a caspase-dependent mechanism. The phospho-p53, Bax and Bad protein expressions in clone 9 cells were increased after TAA treatment.

CONCLUSION: These results reveal that TAA activates p53, increases caspase 3, Bax and Bad protein contents, perhaps causing the release of cytochrome c from mitochondria and the disintegration of membranes, leading to apoptosis of cells.

INTRODUCTION

Thioacetamide (TAA) is employed as a curing ingredient, a chemical reagent, a raw medicine, a pesticide, a textile dye and a finishing auxiliary. TAA^[1-4] is a typical hepatotoxin that causes centrilobular necrosis similar to carbon tetrachloride^[5-8] and D-galactosamine^[9]. TAA-induced hepatotoxicity *via* its S-oxide metabolite (thioacetamide-S-dioxide), that interferes with the motion of RNA from the nucleus to the cytoplasm, resulting in structural and functional cellular deformation and leading to membrane injury. TAA induces hepatocyte damage following its metabolism to thioacetamide sulphene and sulphone, *via* a critical pathway that involves CYP450E1-mediated biotransformation^[10,11]. TAA is a well-known hepatotoxicant whose administration to rodents *in vivo* causes cell death *via* both apoptosis and necrosis^[2,12,13]. The observed liver enzyme activities of aspartate and alanine aminotransferases (AST, ALT), glutamate dehydrogenase and threonine deaminase were low in the TAA-treated group. The declines were significant for both transaminases and threonine deaminase^[14]. TAA (200 µg/mL)-incubated hepatocyte cells exhibited a 40%-62% reduction in the marker enzymes (AST, ALT, and alkaline phosphatase (AP)) and a 50%-61% reduction in both viability and O₂ uptake^[15]. It may reduce the amount of antioxidant reagents, including vitamin C, vitamin E and glutathione contents^[16].

Elevated levels of reactive oxygen species (ROS) are believed to mediate damage by their interaction with proteins, nucleic acids, carbohydrates and lipids^[17-19], changing the enzymatic function and forming oxidation products. ROS is maintained at physiologically optimal levels under normal conditions by antioxidant defense systems that contain nonenzymatic antioxidants-glutathione and enzymes such as superoxide dismutases

(SOD), catalase and glutathione peroxidase (GPX)^[20]. The reduction in the antioxidant defenses and the reduced scavenging capacity reported herein may contribute to oxidative stress in rats with hemorrhagic shock and can be explained by various factors. The observations of apoptosis in liver are based on histochemical changes^[2], the participation of ROS^[21] and lipid peroxidation^[22-24], and cysteine-aspartate proteases3 (caspase 3) activation^[16,25] following the administration of TAA. However, the mechanisms of TAA-induced liver injury are not yet completely understood. The rat liver cell line, clone 9, is an epithelial cell line isolated from a young male rat in 1968 and has primarily been used for studies of *in vitro* carcinogenesis and toxicology. This study was to investigate whether TAA-induced oxidative stresses caused cell death and to seek the pathway that is involved in TAA-induced apoptosis using the rat hepatic epithelial cell line clone 9 as a model and to understand the regulatory mechanisms of TAA-induced liver injury.

MATERIALS AND METHODS

The rat hepatic epithelial cell line clone 9 (CRL-1439, ATCC, USA) was cultured with DMEM/F-12 medium to which 10% fetal bovine serum was added in 50 ml/L CO₂ at 37°C.

Experimental treatments

In Expt1, 5×10^5 clone 9 cells were cultured for 12-16 h in 6 wells and the medium was changed. Afterward, 25, 100 or 200 mg/L TAA was added to the wells and incubated for various times to determine the cell viability (MTT assay). In order to get a large enough cell pellet for chemical assay, the density of cultured cells was changed to 2×10^6 and cells were cultured in 25 cm² flasks. In Expt2, 2×10^6 clone 9 cells were cultured for 12-16 h in 25 cm² flask and the medium was changed. Then, 100 mg/L TAA was added to the flask and incubated for various periods to measure caspase 3, Bad, Bax and Phospho-P53 contents (Western blot), as well as total GSH and caspase 3 activity. In Expt3, 2×10^6 clone 9 cells were cultured for 12-16 h in a 25 cm² flask and the medium was changed. Thereafter, 100 mg/L TAA was added to the flask and incubated for various times to examine DNA fragmentation (TUNEL assay) and measure the proportion of apoptosis (flow cytometry).

MTT assay

750 μ L of MTT (5 mg/mL, Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) solution was added to each well and incubated for 4 h. 1 mL of a DMSO working solution (180 mL DMSO with 20 mL 1 N HCl) was added to each well, and the OD of the yellow reaction product was evaluated in an ELISA reader at a wavelength of 570 nm with a reference wavelength of 630 nm after 15 min.

TUNEL assay

DNA strand breaks were identified using the Boehringer TUNEL assay (Boehringer Mannheim, Marburg, Germany). Briefly, cells in each experiment were washed

with phosphate-buffered saline (PBS). The cells were fixed and permeabilized using the Starfix IQP-200 reagents (Immuno Quality Products, Groningen, The Netherlands) according to the manufacturer's instructions. Subsequently, the TUNEL reaction mixture (60 min, 37°C) was added and the samples were washed and analyzed using FACSsort flow cytometry (Becton Dickinson, New Jersey, USA). The cells that expressed FITC fluorescence were considered to be TUNEL positive. This experiment was performed separately: the observed numbers of apoptotic cells could not be compared directly with those counted in the other experiments. Additionally, this experiment was conducted after a culturing period of 48 h.

Caspase 3 activity assay

The caspase 3 assay was performed according to the method of Hampton *et al*, 2002^[26]. Briefly, cells were treated with lysis buffer (100 mmol/L Hepes, 10% sucrose, 5 mmol/L dithiothreitol and 0.1% Chaps at pH 7.25). The extracted lysates were incubated with DEVD-p-nitroaniline for 3 h at 37°C. Caspase 3-dependent cleavage of p-nitroaniline (pNA) was monitored at 405 nm by spectrophotometry. Caspase 3 activity is expressed in pmol/min per μ g protein.

Glutathione assay

Total cellular GSH was measured using a slight modification^[27,28] of the Tietze assay^[5]. The Tietze assay is a sensitive and specific method for determining the amounts of both reduced (GSH) and oxidized (GSSG) forms of glutathione in unknown samples^[28]. A stock buffer solution of 0.1 mmol/L sodium phosphate, pH 7.5, with 1 mmol/L EDTA was prepared in distilled water and utilized to make separate solutions of 12 mmol/L NADPH, 0.1 mmol/L DTNB and 50 U/mL GSH reductase. The assay was carried out directly in 96-well plates. 100 μ L DTNB, 20 μ L GSH reductase and 20 μ L NADPH were added to each well. Absorbance at 405 nm with a reference wavelength of 595 nm was measured for 30 min at room temperature, using an automatic multiwell microplate spectrophotometer (Beckman, Opsys, MR, USA), and final values were calculated as a mean of three readings. The automatic mix function on the microplate reader was utilized to shake the plate before each reading. Standards of known GSSH content were prepared by serial dilution in 1x MES buffer and used to construct a standard curve. The rate of increase in absorbance at each concentration of GSSH standard was linear throughout the 1.5 min assay. The total protein concentration of each sample was determined using a Bio-Rad kit^[29] with bovine serum albumin as standard, such that GSH levels could be expressed as μ mol per μ g protein. All determinations were carried out in multiples of 12 wells.

Western blotting

After treatment, the cells were washed in D-PBS and lysed with protein lysis buffer (137 mmol/L NaCl, 20 mmol/L Tris-HCl, pH 7.4, 1 mmol/L CaCl₂, 1 mmol/L MgCl₂ and 0.1 mmol/L sodium orthovanadate, containing 1% Nonidet P-40 and 1 mmol/L phenylmercuric sulfanyl chloride). The lysate was centrifuged at 12000 r/min for 10 min at 4°C and the supernatant was collected. The protein

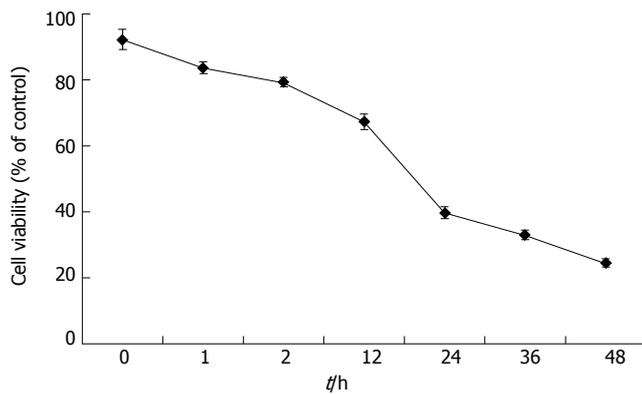


Figure 1 Cell viability of clone 9 after treatment with 100 mg/L TAA for various periods. The results were obtained using MTT assay. Data are presented as mean \pm SE. Individual experiment was repeated three times and each time point of treatment was triplicate.

content was determined using Bradford assay (BioRad) with BSA as a standard. The supernatant (total protein 20 μ g) was mixed with 6x electrophoresis sample buffer that contained 1, 4-dithiothreitol (DTT). The proteins were separated by electrophoresis on a 10% gradient polyacrylamide slab gel and were electrophoretically transferred to a PVDF membrane (Amersham Life Science, Buckinghamshire, UK). The blots were incubated overnight in 3% BSA/TBS-T buffer (50 mmol/L Tris-HCl, 2.7 mmol/L KCl, 0.01 mol/L phosphate, 0.09% NaCl, pH 7.5 and 0.05% Tween-20) for 2 h. Membranes were incubated for 12-16 h with rabbit anti-caspase 3 polyclonal antibody (in a 1:1000 dilution, MWt 35 kDa, Cat. #9665, Cell Signaling, MA, USA), rabbit anti-Bad polyclonal antibody (in a 1:2000 dilution, MWt 21.5 kDa, Cat. #9292, Cell Signaling), rabbit anti-Bax polyclonal antibody (in a 1:1000 dilution, MWt 20.5 kDa, Cat. #2772, Cell Signaling) or mouse anti-Phospho-P53 monoclonal antibody (in a 1:2000 dilution, MWt 53 kDa, Cat. #9286S, Cell Signaling). After three washes with PBST, the blot was incubated with AP-conjugated secondary antibody for at least 2 h and immunoreactive proteins were visualized using BCIP/NBT (ZYMED, 00-2209). The band intensities of the control and the TAA treatment were determined using PHORETIX (Feng Jin Biomedical & Instruments Co Ltd, 61397/28052 Memo-HASP1) and the TAA treatment was compared with the control following-actin normalization.

Statistical analysis

All data are presented as mean \pm SE. For each measurement, data obtained at different times were compared statistically ($P < 0.05$) by one-way analysis of variance (ANOVA) with a Duncan multiple range test from SAS/STAT for multiple comparisons. A P value of less than 0.05 was considered to indicate a significant difference.

RESULTS

Cell viability

To determine the effective concentration of TAA, various concentrations of TAA were added to the clone 9 cell line, respectively. The cell survival rate fell to 67% and

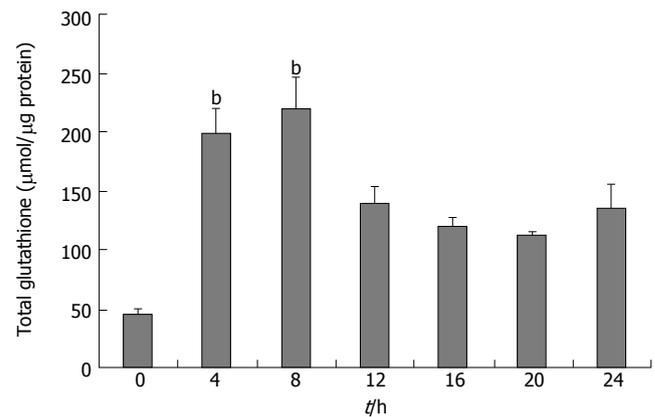


Figure 2 Total glutathione (GSH and GSSG) of clone 9 after treatment with 100 mg/L TAA for various times. Data are presented as mean \pm SE. ^b $P < 0.001$ vs the control (0 h). Individual experiments were repeated three times and each time point of treatment was triplicated.

40% at 12 and 24 h after 100 mg/L TAA-treated clone 9 was applied, respectively (Figure 1). Treatment with a low concentration (25 mg/L) of TAA did not cause significant cell death at 48 h (data not shown). Nevertheless, 200 mg/L TAA was too toxic for clone 9 cells and most cell death occurred within 2 h of treatment. Thus, 100 mg/L TAA was chosen for the subsequent experiments.

Total GSH content

The clone 9 cells were treated with 100 mg/L TAA for various times (4, 8, 12, 16, 20, and 24 h). The total glutathione (GSH plus GSSG) of cell homogenate was measured. The total GSH content of the 100 mg/L TAA-treated clone 9 cells was elevated at 4 h and reached a peak level at 8 h ($P < 0.05$). Afterward total GSH content of the 100 mg/L TAA-treated clone 9 cells declined from 12 h to 24 h compared to that of the TAA-treated 4 h (Figure 2).

Caspase 3

The clone 9 cells were treated with 100 mg/L TAA for various times (4, 8, 12, 16, 20, and 24 h). The caspase 3 activity and protein content (Western blot) of cell homogenate were determined. The caspase 3 activity of the 100 mg/L TAA-treated clone 9 cells reached a peak between 8 and 12 h (Figure 3A). The caspase 3 protein content of the 10 mmol/L TAA-treated clone 9 cells was gradually elevated from 4 h and reached a peak between 8 and 16 h (Figure 3B).

DNA fragmentation

The cell morphology was changed and irregularly shaped DNA was distributed close to the interior of the cell membrane, appearing like an apoptotic body (Figure 4A). Hoechst 33342 and TUNEL assay were applied to elucidate the type of cell death after TAA-treated clone 9 cells. The occurrence of apoptosis increased by 23% at 8 h after 100 mg/L TAA-treated clone 9 cells (Figure 4B).

Phospho-P53 expression

The clone 9 cells were treated with 100 mg/L TAA for various times (4, 8, 12, 16, 20, and 24 h). The phospho-P53

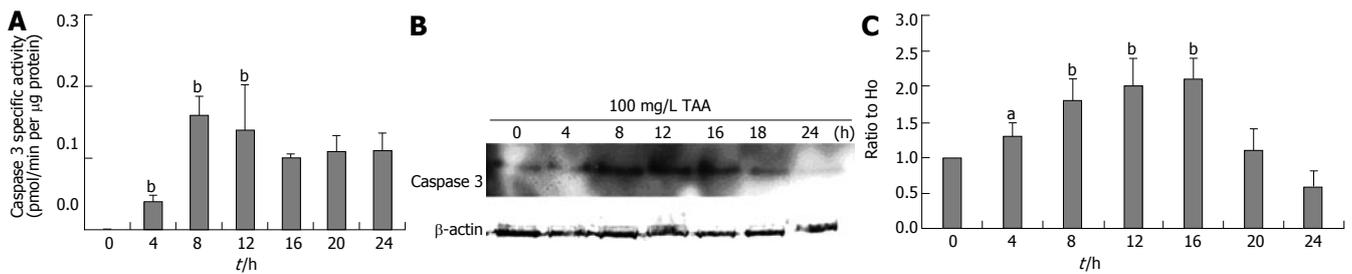


Figure 3 A: Caspase 3 activity; B: Caspase protein level (Western blot); C: Alterations of caspase protein compared to H0 (zero hour) after β-actin normalization (Western blot) in clone 9 after treatment with 100 mg/L TAA for various times. Data are presented as mean ± SE. ^a*P* < 0.05 vs the control (0 h); ^b*P* < 0.01 vs the control (0 h). Individual experiments were repeated three times and each time point of treatment was triplicate.

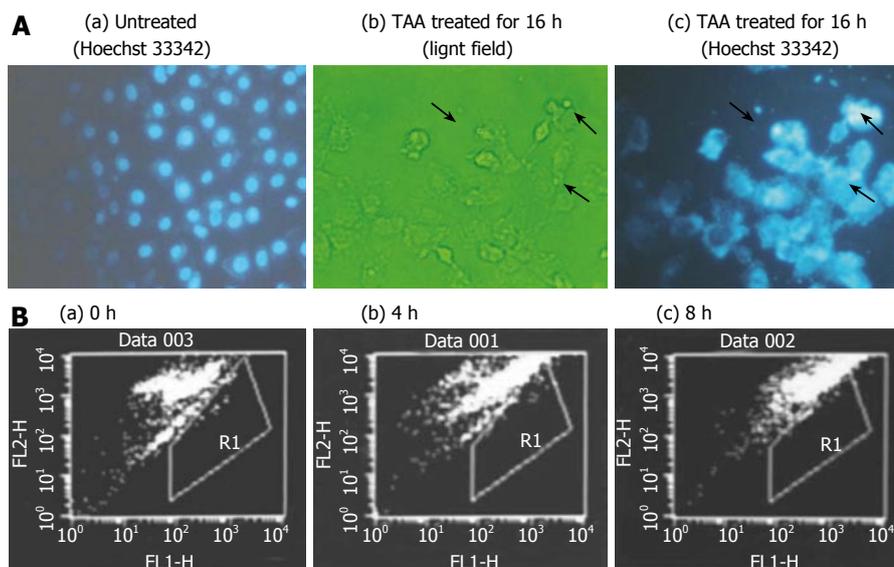


Figure 4 A: Morphology of clone 9 cell; (a) untreated (Hoechst 33342 staining); (b) after treatment with 100 mg/L TAA treatment for 16 h (light field); (c) after treatment with 100 mg/L mmol/L TAA for 16 h (Hoechst 33342, 5 mg/mL in PBS). The arrow indicates the apoptotic cells; B: Flow cytometry of apoptosis in clone 9 cells (TUNEL assay) after treatment with 100 mg/L TAA for various times (a) 0 (b) 4 and (c) 8 h. R1 presents the area of cell apoptosis. Individual experiments were repeated three times and each time point of treatment was triplicate.

protein (Western blot) of cell homogenate was determined. The phospho-P53 expression of 100 mg/L TAA-treated clone 9 cells was increased at 4 h and maintained to 8 and 12 h (*P* < 0.05) (Figure 5).

Bax and Bad expressions

The clone 9 cells were treated with 100 mg/L TAA for various times (4, 8, 12, 16, 20, and 24 h). The Bax and Bad protein (Western blot) of the cell homogenate were determined. The Bax and Bad expressions of 100 mg/L TAA-treated clone 9 cells were elevated at 4 h (*P* < 0.05) and maintained to 8 and 12 h (*P* < 0.05) (Figures 6 and 7). These results were consistent with the phospho-P53 expression.

DISCUSSION

In the present study, rat hepatic epithelial cell line clone 9 was found apoptotic following TAA-induction.

Total GSH

The binding of the reactive compound thioacetamide-S dioxide to tissue macromolecules may be responsible for hepatic necrosis, apoptosis^[16], perturbations of mitochondrial activity^[30,31] and the elevation of serum cytokine levels. Several studies of rats and cultured cells

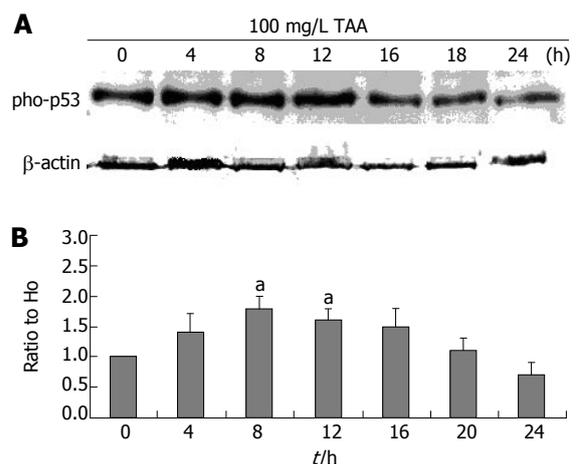


Figure 5 A: Phospho-p53 protein level (typical data, Western blot); B: Alterations of phospho-p53 protein as compared to H0 following β-actin normalization of clone 9 after treatment with 100 mg/L TAA for various times. Data are presented as mean ± SE. ^a*P* < 0.05 vs the control (0 h). Individual experiments were repeated three times and each time point of treatment was triplicate.

have shown the involvement of oxidative stress in the etiology of TAA-induced liver damage. In these works, TAA caused lipid peroxidation^[29,32-35]; increased the susceptibility of hepatocytes to *in vitro* lipid peroxidation^[23]; reduced the GSH/GSSG ratio^[23,34,36,37]; increased GSH

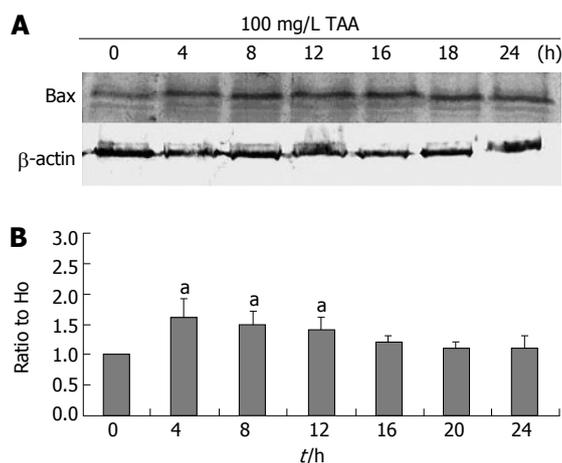


Figure 6 A: Bax protein level (typical data, Western blot); B: Alterations of Bax protein as compared to H0 following β -actin normalization of clone 9 after treatment for various times with 100 mg/L TAA. Data are presented as mean \pm SE. ^a $P < 0.05$ vs the control (0 h). Individual experiment was repeated three times and each time point of treatment was triplicate.

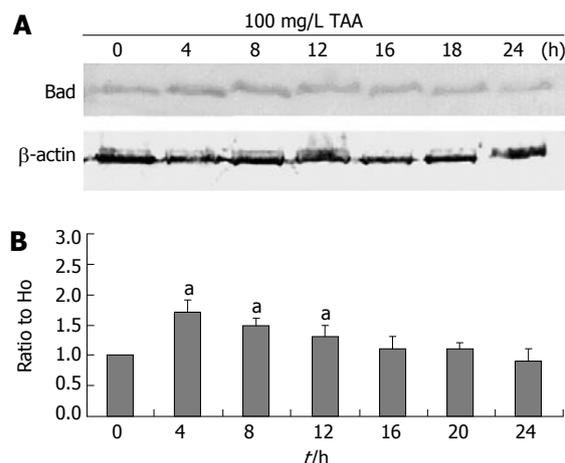


Figure 7 A: Bad protein level (typical data, Western blot); B: Alterations of Bad protein as compared to H0 following β -actin normalization of clone 9 after treatment for various times with 100 mg/L TAA. Data are presented as mean \pm SE. ^a $P < 0.05$ vs the control (0 h). Individual experiments were repeated three times and each time point of treatment was triplicate.

synthesis^[33], alterations in low molecular weight^[16] and the formation of enzymatic antioxidants^[36-38]. The data herein revealed that the total GSH content of 100 mg/L TAA-treated clone 9 cells was elevated at 4 h, reached the highest level at 8 h and decreased from 12 to 24 h (Figure 2). The method in this work detects the total level of GSH. It addresses not only the origin of intracellular GSH and GSSG but also that of the new generation of GSH. Accordingly, total GSH represents the level of cell to anti-oxidative stress. TAA induces oxidative stress, causing the hepatocytes to have a high total GSH content.

Dependence of apoptosis on caspase 3

The discovery of a family of cysteine-aspartate proteases (caspases) and their participation in signaling and apoptosis indicates the critical importance of these enzymes in this form of cell death^[39]. When cells are caused to undergo apoptosis, caspases—in particular caspase 3—cleave ICAD to dissociate the CAD: ICAD complex, allowing CAD to cleave chromosomal DNA^[40]. This study tested caspase 3 protein expressions by western blotting and detected caspase 3 enzyme activity to determine the time point of hepatic apoptosis with TAA treatment. The data herein demonstrate that enzyme activity and western blotting both indicated that caspase 3 activity and the protein of caspase 3 in the 100 mg/L TAA-treated clone 9 cells peaked between 8 and 12 h. The apoptosis caused by TAA involved the activation of caspase 3.

Phospho-p53 and Bax/Bad

In the signaling process associated with apoptosis, the membrane integrity of mitochondria plays an important role and is regulated by the Bcl-2 protein. On the basis of structural and functional attributes, Bcl-2 proteins can be divided into three subgroups; (1) anti-apoptotic channel-forming Bcl-2 proteins with four BH domains (BH1 to -4) and a transmembrane anchor sequence, (2) proapoptotic channel-forming proteins with all but the BH4 domain (Bax, Bak and Bok) and (3) proapoptotic

ligands (BAD, BOD/Bim and BID) that contain only the BH3 domain^[41-43]. The first two subgroups of proteins are believed to be anchored on the mitochondrial membrane, while the third subgroup of proteins act as ligands that dimerize with the membrane-anchored, channel-forming Bcl-2 “receptors”^[44,45]. The BH3 domains in the third subgroup are critical for the binding activity of these ligands. Proapoptotic BOK and BAX with a channel-forming domain regulate apoptosis by releasing APAF-1 because of suppression by anti-apoptotic proteins and by promoting the release of cytochrome c^[46]. Most studies that compare Bax and cytochrome c proteins in cytosolic and mitochondria demonstrate the results of Bax translocation^[47]. In this work, after TAA treatment, the total protein levels of Bax and Bad are elevated perhaps because the regulation of Bax genes increases the amount of Bax protein and the translocation into mitochondria, disintegrating the membrane and causing cell death. The increment of cytosolic Bad protein may be caused by phospho-Bad dephosphorylation; the dephosphorylated Bad and Bcl-XL then generates a dimer that influences the release of cytochrome c and, consequently, apoptosis^[48]. DNA damage activates latent tetramers of p53 to bind in a loosely defined DNA recognition sequence within target gene regulatory elements; p53 then typically activates gene transcription^[49,50]. The activation of DNA binding and subsequent transactivation activities of p53 occur *via* a phosphorylation-acetylation cascade^[51]. The carboxyl-terminus of p53 is phosphorylated in response to DNA damage, as is the amino-terminus; the effects of the amino- and carboxyl-terminal modifications on p53 determine its downstream specificity. Overall, the combination of different phosphorylation and acetylation events that affect p53 tetramers probably determine binding preferences of the downstream target gene-response elements, causing DNA damage-induced signaling *via* p53 phosphorylation. In this work, the expression levels of phospho-p53 and Bax are increased 4 h after treatment with TAA. The apoptotic rate reached 23% at 8 h after treatment with

TAA. This difference may be caused by the activation by ROS of p53. The formation of free radicals and the increase in ROS by H₂O₂ constitute the mechanism of apoptosis^[52-57]. The H₂O₂-induced apoptosis penetrates the cell membrane^[58] and alters Ox-red and free OH-damaged DNA^[59].

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CLINICAL RESEARCH

Wireless capsule endoscopy in the investigation of patients with chronic renal failure and obscure gastrointestinal bleeding (preliminary data)

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group of patients.

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Abstract

AIM: To investigate the role of wireless capsule endoscopy (WCE) in detection of small bowel (SB) pathology in patients with chronic renal failure (CRF) and obscure bleeding.

METHODS: Consecutive CRF patients with obscure bleeding were prospectively studied. Patients with normal renal function and obscure bleeding, investigated during the same period with WCE, were used for the interpretation of results.

RESULTS: Seventeen CRF patients (11 overt, 6 occult bleeding) and 51 patients (33 overt, 18 occult bleeding) with normal renal function were enrolled in this study. Positive SB findings were detected in 70.6% of CRF patients and in 41.2% of non-CRF patients ($P < 0.05$). SB angiodysplasia was identified in 47% of CRF patients and in 17.6% of non-CRF patients. Univariate logistic regression revealed CRF as a significant predictive factor for angiodysplasia ($P < 0.05$). Therapeutic measures were undertaken in 66% of the patients with the positive findings.

CONCLUSION: According to our preliminary results, SB angiodysplasia was found in an increased prevalence among CRF patients with obscure bleeding. WCE is useful in diagnosis of gastrointestinal pathologies and in planning appropriate therapeutic intervention and, therefore, should be included in the work-up of this

INTRODUCTION

Unexplained gastrointestinal bleeding (overt or occult) and anemia are common complications of advanced chronic renal failure (CRF)^[1,2]. Furthermore, 19% of patients with advanced CRF prior to dialysis and 6% of those on maintenance hemodialysis have hemoccult positive stool^[3]. In some cases, upper and lower gastrointestinal tract endoscopic and imaging studies cannot explain these symptoms and findings and bleeding remains a real diagnostic and therapeutic challenge.

Until recently, small bowel (SB) investigation with the available endoscopic and imaging studies was incomplete. Consequently, there are no studies giving information on the pathology of the entire SB in CRF patients, except for two autopsy studies^[4,5]. In view of the scarcity of information, we conducted this study and used wireless capsule endoscopy (WCE) to evaluate SB pathology in patients with advanced CRF and obscure bleeding. This novel method is well tolerated and allows complete visual investigation of the SB^[6,7]. Moreover, the diagnostic yield of WCE, especially in obscure gastrointestinal bleeding, is significantly higher to that of any other methods, including push enteroscopy, small bowel radiography, computed tomography, magnetic resonance imaging and angiography^[8]. To the best of our knowledge, this is the first endoscopic study to explore the entire SB in CRF patients with obscure gastrointestinal bleeding.

MATERIALS AND METHODS

This prospective ongoing study included consecutive patients referred to our institution with advanced CRF needing SB investigation for obscure bleeding. Advanced CRF was defined as creatinine clearance of less than 30 mL/min. Renal transplant recipients with stable renal function and serum creatinine concentration ≥ 2 mg/dL for at least a 6-mo period were eligible to enter the study as well.

Age- and sex-matched patients with normal renal function and obscure gastrointestinal bleeding, investigated with WCE during the same period, were used for the interpretation of CRF patients' findings.

Obscure gastrointestinal bleeding was defined according to the American Gastroenterological Association position statement^[9]. Patients with obscure bleeding had been initially investigated with esophagogastroduodenoscopy and colonoscopy in other institutions and, if negative, were subsequently referred for WCE.

Generally accepted contraindications for the WCE procedure are described elsewhere^[10]. Written informed consent was obtained from all patients. Patients' clinical characteristics, including sex, age, total duration of CRF (pre-treatment plus post treatment period), NSAID or aspirin use, comorbid diseases (practically any serious diseases, e.g. cardiovascular, collagen diseases, endocrinopathies, portal hypertension), need for previous transfusions as well as the etiology of renal failure were recorded. Biochemical tests of renal function (serum creatinine and urea) and hemoglobin level were obtained as well.

WCE procedure

M2A capsule (Given Imaging, Yoqneam, Israel) was used in this study. Patients' preparation and WCE procedure were carried out following the generally recommended guidelines^[11]. Hemodialysis patients were not given the two-liter solution of polyethylene glycol as preparation, due to restriction of fluid intake; instead, they were advised to abstain from solid food, on the day before the procedure.

Interpretation of WCE results

A single gastroenterologist (experienced in gastrointestinal endoscopy and WCE) initially screened all videos and selected images of potential abnormalities. Then, two gastroenterologists (also experienced in interpreting WCE findings) independently reviewed the selected images to confirm the accuracy of diagnosis. All videos were extensively discussed and findings identified by both reviewers were considered as definitive and included in the report. The procedure was defined as incomplete if the capsule failed to pass into the cecum during an eight and a half-hour period of the examination. The diagnostic yield of WCE was calculated for both CRF and non-CRF patients. Only lesions with a high potential for bleeding, as defined by Saurin *et al.*^[12], were considered.

Statistical analysis

The Statistical Package for Social Sciences (SPSS) program

Table 1 CRF patients' characteristics

No. of patients	17
Etiology of renal failure, <i>n</i> (%)	
Nephrosclerosis	4 (23.5)
Glomerulonephritis	3 (17.6)
Chronic pyelonephritis	1 (5.9)
Diabetic nephropathy	1 (5.9)
Other etiologies	3 (17.6)
Unknown	5 (29.4)
Duration of CRF (mean \pm SE), mo	139.9 \pm 13.0
Median serum creatinine (range), mg/dL	3.2 (2.0 - 9.4)
Serum urea (mean \pm SE), mg/dL	133.9 \pm 11.0

version 10.0 (Chicago, Illinois) was used for statistical analysis. Continuous data with normal distribution were expressed as mean \pm SE, while those without as median (range). Differences between groups were evaluated using χ^2 test for qualitative variables and Student's *t* test for quantitative variables following a normal distribution or the Mann-Whitney *U*-test for those who failed the normality test. A *P* value of less than 0.05 was considered statistically significant. Logistic regression analysis was used to find predictive variables of findings, which were identified in terms of odds ratio (OR) with 95% confidence intervals (95% CI).

RESULTS

A total of 17 CRF patients fulfilled the inclusion criteria (7 predialysis patients, 4 on maintenance hemodialysis and 6 renal transplant recipients). CRF etiology and duration as well as biochemical parameters are shown in Table 1. Duration of dialysis ranged between 20 to 60 mo (mean \pm SE: 38.7 \pm 3.8) and the treatment schedule consisted of 4 h hemodialysis procedures three times weekly. Post-transplantation period ranged between 16 to 93 mo (mean \pm SE: 53.2 \pm 12.5). Immunosuppressive treatment in transplant recipients consisted of a combination of mycophenolate mofetil (MMF), cyclosporine and prednisone (3/6 patients), MMF, tacrolimus and prednisone (2/6) and MMF, sirolimus and prednisone (1/6). Two out of 17 CRF patients were taking low-dose aspirin and 1 non-steroidal anti-inflammatory drug (NSAID).

The group of non-CRF patients consisted of 51 patients with obscure bleeding. Table 2 shows the demographic and clinical characteristics of CRF and non-CRF patients. The two groups were found comparable regarding indications for WCE (overt, occult bleeding), NSAID or aspirin use, comorbid diseases, hemoglobin level and need for previous transfusions.

CRF patients completed the procedure uneventfully. One case of capsule retention was observed in a non-CRF patient with an adenocarcinoma of the mid ileum. In 3 (17.6%) CRF and 10 (19.6%) non-CRF patients, the capsule did not reach the colon, and, therefore, the entire SB was not imaged. Causes of failure of the capsule to reach the colon within the recording time in CRF patients were: slow gastric passage (1 case), presence of food

Table 2 Demographic and clinical characteristics of CRF and non-CRF patients

	CRF patients	Non-CRF patients	P
No. of patients	17	51	
Male/female	12/5	36/15	NS
Age (mean ± SE), yr	57.1 ± 2.5	57.2 ± 2.1	NS
Indication of WCE (overt/occult bleeding)	11/6	33/18	NS
NSAIDs or aspirin use, n (%)	3/17 (17.6%)	14/51 (27.5%)	NS
Comorbid diseases, n (%)	10/17 (58.8%)	23/51 (45.1%)	NS
Hb (mean ± SE), g/L	84.3 ± 2.0	87.7 ± 1.3	NS
Need for previous transfusions, n (%)	6/17 (35.3%)	28/51 (54.9%)	NS

NS: Non-significant.

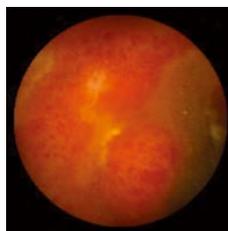
Table 3 Small bowel findings revealed by wireless capsule endoscopy

SB findings	CRF patients n = 17 (%)	Non-CRF patients n = 51 (%)
Angiodysplasia	8 (47.0)	9 (17.6)
Single ulcer	2 (11.8)	3 (5.9)
Ulceration with cobblestoning and stricture	1 (5.9)	3 (5.9)
Non-specific ulcers, erosions, submucosal bleeding, erythema and edema	1 (5.9)	-
Fresh blood without an obvious explanation	-	2 (3.9)
Tumor	-	2 (3.9)
Multiple erosions	-	2 (3.9)
No findings	5 (29.4)	30 (58.8)

impairing the capsule progression (1 case), and no clear reason (1 case). Gastric emptying time in CRF patients ranged from 5 to 288 min (median: 20) and SB transit time from 99 to 393 min (median: 275).

WCE findings of SB in both CRF and non-CRF patients are listed in Table 3. In CRF patients, the most frequent type of SB lesion identified was angiodysplasia (8/17, 47%; two actively bleeding) (Figure 1). Patchy mucosal redness and a big jejunal ulcer (Figure 2) were revealed in 2 patients, 1 of which had a recent history of NSAIDs use. Ulceration, cobblestoning, stricture and a pattern of discontinuous involvement of the mid ileum, suggestive of Crohn's disease, were found in 1 patient. Finally, in 1 patient with erythema, edema, submucosal bleeding, erosions and non-specific ulcers of the distal ileum, caecum and ascending colon (Figure 3), tissue diagnosis of CMV enterocolitis was subsequently made by means of ileocolonoscopy and biopsies. Preceded colonoscopy in another institution, ten days earlier, had missed the lesions. Consequently, the diagnostic yield of WCE in CRF patients with obscure bleeding was significantly higher (70.6%, 12/17) compared with the non-CRF patients (41.2%, 21/51) ($\chi^2 = 4.42, P < 0.05$).

Angiodysplasia was the most frequent finding in the SB of CRF patients. A single lesion was found in 5 patients, 2 lesions in 2 and 3 in 1. Angiodysplasias were located

**Figure 1** Single ileal angiodysplasia revealed by WCE.**Figure 2** Patchy mucosal redness and a big jejunal ulcer detected by WCE.**Figure 3** Wireless capsule endoscopy showing erythema, edema, submucosal bleeding and ulcer of the distal ileum in a renal transplant recipient.

in jejunum (5 patients), ileum (2 patients) and both (1 patient).

We assessed presence of CRF, age, sex, comorbid diseases, hemoglobin level at the time of the procedure and need for previous transfusions as potentially relevant independent variables for angiodysplasia in both CRF and non-CRF patients. Univariate logistic regression revealed only CRF as a significant predictive factor for angiodysplasia (OR = 4.1, 95% CI = 1.3-13.7, $P < 0.05$). Moreover, we evaluated sex, age, duration of CRF and comorbid diseases as potentially relevant independent variables for SB angiodysplasias in CRF patients. SB angiodysplasias seem to be associated only with the duration of CRF (OR = 1.03, 95% CI = 0.99-1.06, $P < 0.1$).

In view of the WCE findings, 4/8 patients with angiodysplastic lesions within the reach of push enteroscopy were referred for endoscopic treatment with argon plasma coagulation and 1 patient with 3 angiodysplasias received estrogen therapy. Pharmacotherapy was provided in the patient with Crohn's disease as well as in the patient with CMV enterocolitis, and discontinuation of NSAIDs in the patient with the big jejunal ulcer was undertaken. Iron supplementation was decided to be the only measure in the remaining 3 cases with angiodysplasias and in the patient with the jejunal ulcer.

DISCUSSION

Gastrointestinal bleeding is a common complication of advanced CRF^[1,2]. Although there have been many studies concerning implication of upper gastrointestinal tract and colon, data concerning SB are limited. WCE is a new method for the investigation of SB pathology, but already has an established role in cases of obscure gastrointestinal

bleeding^[8]. We conducted this study in order to investigate SB pathology in CRF patients with obscure gastrointestinal bleeding and assess the diagnostic and therapeutic yield of the method in this group of patients. To our knowledge, there is only one case report on this issue^[13].

According to the preliminary results of our ongoing study, angiodysplasia was recognized as a sole causative lesion of obscure bleeding in a markedly higher percentage, 47% in CRF patients *vs* 17.6% in non-CRF patients. A high prevalence (66.7%) of SB angiodysplasia among 6 CRF patients has also been reported in a recent study on the impact of WCE in obscure gastrointestinal bleeding^[14]. In our study, logistic regression analysis showed that CRF was a significant predictive factor for SB angiodysplasia (OR = 4.1, 95% CI = 1.3-13.7, *P* < 0.05). Furthermore, our data suggested that the prevalence of angiodysplasia seemed to be inversely related with the duration of CRF, although this finding was statistically insignificant (OR = 1.03, 95% CI = 0.99-1.06, *P* < 0.1). Previous reports on the prevalence of angiodysplasia in CRF patients, as well as its association with patient's age and duration of the disease are conflicting^[15-18]. However, all those studies used data obtained by conventional upper gastrointestinal endoscopy.

The diagnostic yield of WCE in CRF patients with obscure bleeding was significantly higher (70.6%) than that in non-CRF patients ($\chi^2 = 4.42$, *P* < 0.05), which is in agreement with previous data^[19-21]. Given that WCE has already become the method of choice in the investigation of patients with obscure bleeding^[8], its value in the subgroup of CRF patients is even more crucial.

WCE findings led to plan a specific intervention in 8/12 patients with positive findings. Therapeutic measures included push enteroscopy with coagulation of angiodysplasias in 4 patients, pharmacotherapy in 3 and discontinuation of medication in another 1.

In conclusion, according to the preliminary results of our ongoing study, SB angiodysplasia has an increased prevalence among CRF patients with obscure bleeding. WCE is useful in diagnosis of gastrointestinal pathologies and in planning appropriate therapeutic intervention and, therefore, should be included in the work-up of this group of patients.

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RAPID COMMUNICATION

Muscle fatigue in women with primary biliary cirrhosis: Spectral analysis of surface electromyography

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such patients may be of central origin.

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Abstract

AIM: To evaluate the myoelectric manifestations of peripheral fatigability in patients with primary biliary cirrhosis in comparison to healthy subjects.

METHODS: Sixteen women with primary biliary cirrhosis without comorbidity and 13 healthy women matched for age and body mass index (BMI) completed the self-reported questionnaire fatigue impact scale. All subjects underwent surface electromyography assessment of peripheral fatigability. Anterior tibial muscle isometric voluntary contraction was executed for 20 s at 80% of maximal voluntary isometric contraction. During the exercise electromyographic signal series were recorded and root mean square (expression of central drive) as well as mean and median of electromyographic signal frequency spectrum (estimates of muscle fatigability) were computed. Each subject executed the trial two times. EMG parameters were normalized, then linear regression was applied and slopes were calculated.

RESULTS: Seven patients were fatigued (median fatigue impact scale score: 38, range: 26-66) and 9 were not fatigued (median fatigue impact scale score: 7, range: 0-17). The maximal voluntary isometric contraction was similar in patients (82, 54-115 N) and controls (87, 74-101 N), and in patients with high (81, 54-115 N) and low fatigue impact scale scores (86, 65-106 N). Root mean square as well as mean and median of frequency spectrum slopes were compared with the Mann-Whitney U test, and no significant difference was found between fatigued and non-fatigued patients and controls.

CONCLUSION: No instrumental evidence of peripheral fatigability can be found in women with primary biliary cirrhosis but no comorbidity, suggesting that fatigue in

INTRODUCTION

Fatigue affects 60%-80% of patients with primary biliary cirrhosis (PBC), and is considered to be specifically associated with PBC^[1-3]. However, current understanding of fatigue pathophysiology in PBC is limited and comorbidity may play a significant role in fatigue pathophysiology^[4].

The comprehension of this symptom has been hampered by the complexity of its nature and relationships with somatic illnesses, psychological disturbances, and stress reactions. Moreover, fatigue is subjective and difficult to measure^[5]. The most used instruments for measuring fatigue in PBC are self-reported questionnaires such as the fatigue impact scale (FIS)^[3,6-10].

Several studies support the hypothesis that fatigue has a central origin^[10-13]. In contrast, Goldblatt and colleagues^[7] assessed muscle fatigability in PBC patients with a grip strength protocol, and found that patients with high FIS scores are characterized by an accelerated decrease in muscle function compared with both healthy controls and non-fatigued patients.

However grip strength cannot distinguish if muscle fatigability is due to a central or a peripheral dysfunction, and it is not able to detect volitional components. Moreover, Stanca *et al*^[14] have not found any difference in grip strength between patients with high FIS scores and controls. No study has completely excluded a peripheral component of fatigue in PBC patients.

A validated surface electromyographic technique which provides objective measures of peripheral fatigability

has been developed^[15-17]. This method is able to monitor central drive and has been applied in both physiological and pathological conditions, but never in chronic liver diseases^[18-20].

The aim of this study was to investigate if myoelectric manifestations of peripheral fatigability during voluntary isometric contractions in women with PBC, but no comorbidity, are significantly different from those in healthy subjects matched for their age, sex and body mass index (BMI).

MATERIALS AND METHODS

Patients

Fifty-seven patients with PBC were screened for several common diseases (renal failure, anaemia, electrolyte imbalance, thyroid-associated disease, rheumatic fibromyalgia, polyneuropathy and diabetes) for assumption of drugs that could cause fatigue (b-blockers, *etc.*).

A subgroup of 16 women without nonhepatic causes of fatigue underwent EMG assessment of peripheral fatigability. The other patients did not participate in the study because of nonhepatic causes of fatigue or because they denied their consent. All patients were stable with no history of bleeding, ascites or encephalopathy. Laboratory investigations included blood cell count, liver function tests, detection of creatinine, thyroid hormones, HbA1c, iron, serum electrolytes, and anti-mitochondrial antibodies. All patients were treated with ursodeoxycholic acid (UDCA) at the time of the study.

Controls

Thirteen healthy women comparable for age and BMI who declared that they did not assume drugs or suffer from any disease served as controls.

Ethics

The study protocol conformed to the ethical guidelines of the 1996 Helsinki Declaration and all subjects gave their informed written consent to participating in the experimental study.

Questionnaires

All subjects completed two self-reported questionnaires in a proper setting: fatigue impact scale and Rand MOS depression screening. FIS is a multidimensional scale composed of 40 items providing a global score (maximum 160), containing the intermixed physical, cognitive (maximum score 40 each) and psycho-social (maximum score 80) domains of fatigue^[21]. FIS has been recently validated in PBC patients^[22].

Rand MOS depression screening is composed of 8 items to screen the depressive disorders (major depression and dysthymia) and has been used in general and chronic disease populations, and in PBC patients^[23]. It does not consider physical depressive symptoms that can be found also in abdominal diseases.

Experimental protocol

EMG analysis of isometric contractions of the dominant anterior tibial muscle was conducted in this study. This

muscle was chosen because of its simple anatomy and because it has been analyzed by other investigators for fatigue studies^[15].

Subjects laying on a bed were asked to put on a specially designed boot. The ankle joint was fixed with Velcro straps. A force transducer (Digitalanzeiger mod. 9180) was displayed to measure the force produced by the dorsal extension of the feet, and a display located at bed side showed the instantaneous force values expressed in Newton.

Before EMG signal acquisition, the subjects were requested to perform a short (5 s) maximal voluntary isometric contraction three times with a 2 min rest between each contraction. The mean maximal voluntary isometric contraction (MVC) of the three recorded maximal force values was used as the reference value.

Fatigue test for anterior tibial muscle isometric contraction was performed for 20 s at 80% of MVC. This target force was reached and held by means of the visual feedback display located at bed side showing the instantaneous force values. Standardised verbal encouragement was given throughout this force-failure point. Fatigue test was performed two times with 5 min rest between the two sessions.

The following clinical force parameters were considered: MVC and fatigue threshold (FT) which is time necessary to reduce target force more than 10%. The first one could be considered as an estimation of the muscle force, while the second parameter could be considered as the clinical evidence of fatigability.

The skin was cleaned and two pregelled disposable surface electrodes (Bionen Firenze) were placed 20 mm apart on the longitudinal midline of the dominant anterior tibial muscle between the motor point and the tendon. A ground electrode was attached to the knee. EMG signals were recorded during each session of the fatigue test.

Neurophysiologists were blinded to FIS scores of the subjects.

Analysis of data

Twenty series of 500 ms windows were taken from the 20 s data of the EMG signal of the fatigue test. For each window, root mean square (RMS) amplitude of the EMG signal and spectral analysis (512 points, Hanning window processing, fast Fourier transform) were applied. The mean MNF and median MDF values of EMG signal frequency spectrum were computed. EMG parameters were normalized to the values generated at the beginning of each session. Then, linear regression was applied to the time-series of RMS, MNF and MDF to estimate their rate of change (slope of their respective linear regression).

MNF and MDF slopes are an estimate of muscle fatigability^[15]. MNF and MDF tended to decrease during the trial in normal subjects, thus the slope values of their respective linear regression were negative. Subjects with a higher fatigability have greater absolute values of MNF and MDF slopes. RMS is an expression of the central drive and its value tended to increase during the trial, thus the slope value of its linear regression was positive. The analysis of peripheral fatigability was possible only when RMS slope was positive. The finding of a low central drive

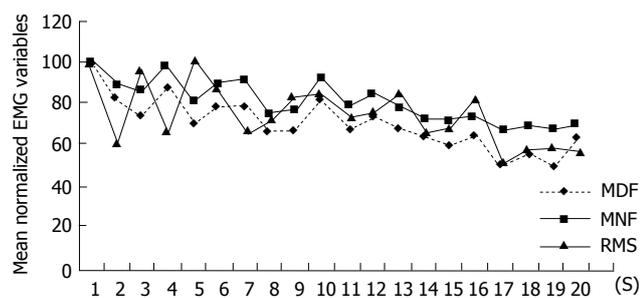


Figure 1 Normalized values of EMG variables during voluntary isometric contractions at 80% MVC from a patient with low central drive. In this case the trial was not included in the analysis of peripheral fatigue.

could be used as an index of the central pathology or it could be due to a volitional component.

Statistical analysis

The Mann-Whitney *U* test (non-parametric test for independent samples) was used to identify whether significant differences in MVC existed between subject groups. Statistical comparison was made between controls and patients, between controls and subgroups of fatigued and non-fatigued patients.

EMG parameters were analyzed during each recording session by repeated measurements (ANOVA). The univariate solution was obtained after correction for the Greenhouse-Geisser factor when appropriate, to protect against type I errors associated with nonsphericity of data. In each case, the approximate *F* value associated with the univariate test was reported. Variables showing significant effects or interactions ($P < 0.05$) were subjected to *post hoc* testing (Scheffé) using an alpha level of less than 0.05. Sample regression of EMG parameters was used to evaluate the inter-observation variation between the first and second session.

All statistical analyses were performed using StatView for Windows.

RESULTS

Clinical features of the 16 selected patients (median age: 54 years, range: 37-73 years; median BMI: 23 kg/m², range: 17.9-28.5 kg/m²) and the controls (median age: 51 years, range: 45-64 years; median BMI: 23.2 kg/m², range: 18.2-28.4 kg/m²) were comparable. Our PBC population was composed of 2 patients in stage I, 6 in stage II, 7 in stage III and 1 in stage IV. Liver biopsies were taken no more than 5 years of post-muscle testing, during which time clinical and laboratory features were not substantially modified.

We divided the patients into two groups according to their FIS scores: seven fatigued (median total FIS: 38, range: 26-66) and nine non-fatigued (low or very low total FIS; median total FIS: 7, range: 0-17). Depression assessed by Rand MOS Depressions Screening was present in two patients.

The MVC force was not statistically different between patients (median: 82 N; range: 54-115 N) and controls (median: 87 N; range: 74-101 N). No difference was found also between patients with high (median: 81 N; range:

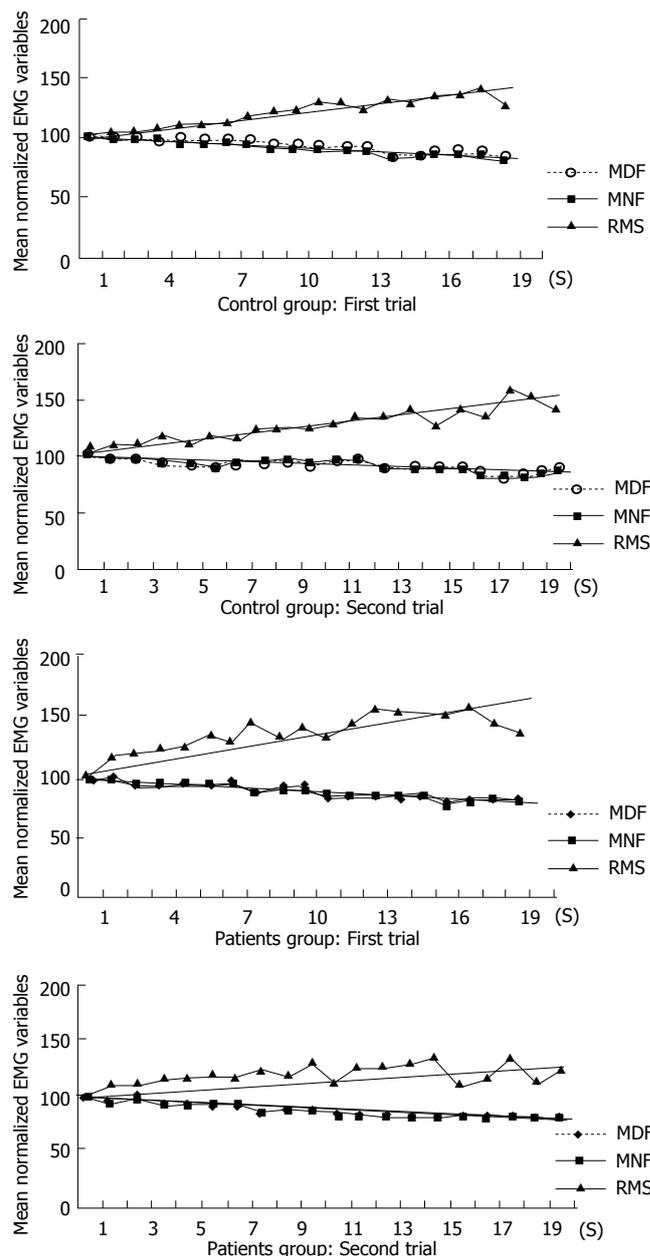


Figure 2 Mean normalized values of EMG variables during voluntary isometric contractions at 80% MVC from patients and controls. The time course of MNF, MDF, RMS and force were combined to produce a “fatigue plot”. Plots were obtained by normalizing each variable with respect to the initial value of its own regression line.

54-115 N) and low FIS scores (median: 86 N; range: 65-106 N). Fatigue threshold was reached only in two patients.

RMS did not increase during the trial in two patients (Figure 1), so EMG analysis of peripheral fatigability was performed on the remaining 14 patients. These two patients reached the fatigue threshold. Fatigue testing was performed twice in each patient, consequently 28 slope values of each parameter for patients and 26 for controls were obtained.

The mean normalized values of all variables (MNF, MDF, RMS) during voluntary isometric contractions at 80% MVC from patients and controls are shown in Figure 2. RMS ($P = 0.84$), MNF ($P = 0.66$) and MDF ($P = 0.4$) slopes were not significantly different between fatigued

and non-fatigued patients and controls, as well as between the two groups of patients. Inter-observation variation between the first and second sessions was as follows: $r = 0.88$, $P < 0.01$ for MDF; $r = 0.93$, $P < 0.01$ for MNF; $r = 0.80$, $P < 0.05$ for RMS.

DISCUSSION

Fatigue is an overwhelming sense of tiredness, lack of energy or feeling of exhaustion. It is frequently present even at rest. Fatigue is a multidimensional symptom caused by the interaction of physical, psychosocial and cognitive factors. It cannot be confused with weakness or fatigability. The last one in particular is a generalised sense of exhaustion, not present at rest, affecting the patient after a few minutes of physical activity and disappears after a short rest^[24].

Fatigue muscle arises only when muscle fibres are sufficiently activated and consequently develop an effective contraction. Stimulation can be obtained artificially with electrodes or through the physiologic voluntary nervous pathways. The first method gives a more constant elicitation, however it is not physiologic and it is quite an irritation for patients. The second one employs the will of the subjects in keeping the contraction of the analyzed muscle. In our study, isometric contraction of the dominant anterior tibial muscle was voluntary.

Fatigue can be evaluated using a particular EMG analysis. In fact, when muscle fibres contract, they generate electric activities which are recordable with EMG electrodes. EMG signals have different amplitudes and frequencies. The sum of all signals generated by all muscle fibres during the contraction provides a frequency spectrum. As the contraction carries on and the fatigue arises, the frequency spectrum changes. In particular, the mean and median frequency spectrum values (MNF and MDF) decrease proportionally with the duration of contraction and the fatigue of muscle.

As fatigue increases, the subjects effort in keeping a target force increases as well. This effort corresponds to an increase of the stimulation of muscle fibres, in other words we have a higher central drive. The increment of central drive determines the increase of amplitude of the frequency spectrum. Thus EMG analysis of the frequency spectrum can evaluate also the central drive of the subjects during the isometric contraction. Root mean square is an estimate of the amplitude of frequency spectrum, and its value tends to increase during exercise. In our study, as in others, this parameter was used as an estimate of central drive^[15].

When a subject develops fatigue and is not able to keep a target force, it can be due to an inadequate force generation by muscle fibres or by a lack of central drive (peripheral and central fatigue)^[25]. If muscle fibres are not sufficiently stimulated because of a lack of central drive, they do not contract enough to develop fatigue, thus in this condition peripheral fatigue cannot be evaluated.

Central drive is not sufficient for two main reasons: the subject is not motivated and does not make enough effort to execute an adequate muscle contraction and the subject is affected by a pathologic neurological central condition that does not allow activation of the muscle. Hence

peripheral fatigue can be analyzed only in the presence of an adequate central drive, and central drive can be low for a lack of motivation or for a neurological pathology.

To the best of our knowledge, this is the first study in patients affected by PBC in which it has been used as an EMG technique able to quantify fatigability and analyse the presence of peripheral dysfunction.

In our population MVC values were similar in patients and controls. These findings agree with those of Goldblatt *et al*^[7] and we can suppose that PBC patients are not weaker than the general population. In addition, fatigue testing showed that fatigability in women affected by PBC without comorbidity was not significantly different from that in healthy controls matched for age, sex and BMI. Moreover, we did not find any significant difference in peripheral fatigability between fatigued and non-fatigued patients as assessed by FIS.

Two patients had a defective central drive as shown by a flat RMS slope and both of them had high FIS scores and were not able to maintain the 80% MVC target (Figure 1). We do not know if this lack of central activation was due to a volitional component or to a central dysfunction. In fact, our EMG technique was not able to analyze central dysfunction. These two patients had a normal MVC and standard EMG did not show any pathological neuromuscular abnormality. Interestingly, both women were positive for Rand MOS Depression Screening. So, according to the previous studies showing a positive correlation between depression and fatigue, we may suppose a lack of motivation in these two patients^[2,3,6]. If they would have been analysed only with force recording, as done in previous studies, they would be classified as subjects with high muscle fatigability^[7].

In conclusion, no evidence of peripheral fatigability in PBC patients without comorbidity is demonstrable. If these data will be confirmed, fatigue pathogenesis in PBC patients may be related to a central mechanism. This central alteration may be due to a common genesis with the liver disease, or in alternative to one or more causes not related to the hepatic damage (specific or not specific symptom of PBC). Some data support the last hypothesis, in fact two recent reports have failed to demonstrate a significant difference in FIS scores between patients and controls and no correlation has never been found between fatigue and parameters of disease severity and activity^[2,4,6,9,14]. Future research should be addressed on the investigation of central fatigue and the role of depression, which is the only parameter significantly associated with fatigue in several studies^[2,3,6].

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Microheterogeneity of acute phase proteins in patients with ulcerative colitis

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ogeneity patterns of AGP and ACT are similar in ulcerative colitis patients and healthy subjects.

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Key words: Glycoproteins; Acute phase response; Ulcerative colitis

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Abstract

AIM: To estimate the serum α_1 -antichymotrypsin (ACT), α_1 -acid glycoprotein (AGP) and transferrin (Tf) concentrations and to evaluate the microheterogeneity of these acute phase proteins in patients with ulcerative colitis.

METHODS: Twenty-seven patients with ulcerative colitis (UC) and 17 healthy control subjects were studied. The patients were categorised as severe ($n = 9$), moderate ($n = 10$) and mild groups ($n = 8$) using Truelove and Witts' classification of ulcerative colitis. Microheterogeneity of ACT, AGP and Tf was analysed by crossed immunoaffinity electrophoresis (CIAE) with concanavalin A. In all serum samples standard electrophoresis of serum proteins was performed, iron (Fe) concentration, total iron binding capacity (TIBC) and C-reactive protein (CRP) were also measured.

RESULTS: Our patients suffering from ulcerative colitis had significantly higher serum ACT and AGP concentrations and lower serum transferrin concentration in comparison to healthy subjects. Changes in concentrations of acute phase proteins were dependent on the activity of the inflammatory process. The glycosylation patterns of transferrin were related to the inflammation status. We also observed the correlation between ACT and AGP concentrations, patterns of transferrin glycosylation and changes in standard protein electrophoresis or blood cell count.

CONCLUSION: The glycosylation patterns of transferrin obtained from patients suffering from ulcerative colitis are highly branched and sialylated compared with those obtained from healthy subjects. In contrast, the glycosylation patterns of transferrin do not differ according to the activity index of ulcerative colitis. The microhetero-

INTRODUCTION

In ulcerative colitis, a chronic inflammatory disease of unknown etiology, proinflammatory cytokines, such as tumor necrosis factor (TNF), interleukin-1 (IL-1), IL-6 and IL-2, play a major pathophysiological role^[1-5]. Acute phase response is a systemic answer to mechanical injuries, infections as well as an unspecific inflammation. Acute phase proteins (APP) play an important role in this non-specific immune reaction. These proteins are produced by the liver in answer to cytokine mediation. The concentrations of many APP have been studied in ulcerative colitis patients^[6-8]. Measurement of APP serum concentrations offers a valuable means of assessing the intensity of inflammatory bowel disease. Unsurprisingly, various studies have shown the correlation between these inflammatory mediators and acute phase proteins, such as C-reactive protein (CRP), α_1 -acid glycoprotein (AGP), α_1 -antichymotrypsin (ACT) and serum amyloid A (SAA)^[9-11]. A acute phase proteins and clinical signs are also of a high value for the treatment and remission of ulcerative colitis. However, the etiological and clinical importance of these proteins is still not completely revealed.

Except for CRP, albumin and SAA, all acute phase proteins are glycoproteins. Glycosylation is the primary cause of microheterogeneity in proteins. It is an enzyme-directed and site-specific process. Protein sugar prints are conserved, not random and determined independently from the synthetic rate of protein. There is O-linked glycosylation to the hydroxy oxygen of serine and threonine side chains and N-linked glycosylation to the amide nitrogen of asparagine side chains. The N-glycan chains of α_1 -AGP, α_1 -ACT and Tf differ in their branching, showing

Table 1 Clinical characteristics of patients with UC (mean \pm SD)

Trulove and Witts' classification	UC (n = 27)	Severe (n = 9)	Moderate (n = 10)	Mild (n = 8)	P
Female (n)	16	4	6	6	
Male (n)	11	5	4	2	
Age (yr)	41.80 \pm 18.00	40.20 \pm 20.20	38.00 \pm 16.00	48.40 \pm 18.30	NS
Hemoglobin (g/dL)	12.01 \pm 2.21	11.17 \pm 1.92	11.88 \pm 2.14	13.11 \pm 2.38	< 0.05
PLT (thsd/mm ³)	352.00 \pm 190.62	428.78 \pm 116.65	358.90 \pm 235.76	257.00 \pm 173.18	< 0.05
Fe (μ g/dL)	73.90 \pm 35.60	52.33 \pm 19.20	73.20 \pm 26.71	99.00 \pm 45.19	< 0.05
WBC (thsd/mm ³)	8.68 \pm 3.99	10.20 \pm 5.61	9.03 \pm 3.13	6.58 \pm 1.52	< 0.05
Total protein (g/dL)	6.15 \pm 0.99	5.72 \pm 1.20	6.52 \pm 0.71	6.20 \pm 0.93	< 0.05
Albumin (g/dL)	3.22 \pm 0.82	2.70 \pm 0.78	3.63 \pm 0.59	3.40 \pm 0.89	< 0.05

bi-, tri-, and tetra-antennary structures^[12-14]. These serum N-glycoproteins physiologically occur in few variants, called microheterogeneity. During acute phase response both concentrations and percentage of individual variants are changed.

Glycosylation may play a role in cell-cell adhesion. The highly branched and sialylated form of AGP which is the ligand for cell adhesion molecules such as E-selectin and P-selectin, inhibits migration of neutrophils, monocytes and T-cells, and ameliorates complement activity^[15]. The asialylated carbohydrate-deficient variant of AGP appears mainly in sera of patients after acute inflammation, infection, burn or other severe tissue damage or necrosis^[16]. The immunomodulatory properties of AGP have been shown to depend on its glycosylation. The inhibition of lymphocyte proliferation depends on the branching degree of the glycans of AGP^[17]. The sialylated acute phase proteins protect against immune complex-induced injury^[18]. An increase of sialyl-variants of APP seems to be a mechanism responsible for feedback inhibition of leucocyte migration to inflamed tissues. The heterogeneity of the glycosylation pattern of AGP that has been found in patients with ulcerative colitis reflects changes in response to inflammation^[19].

The changes in patterns of glycosylation of transferrin have been observed in iron deficiency anaemia, rheumatoid arthritis, liver cirrhosis or in physiological status such as pregnancy^[20]. The microheterogeneity pattern of Tf shifts under inflammatory conditions towards highly branched glycans. Differences in glycosylation of Tf seem to alter the iron metabolism.

The aim of this study was to estimate the changes in microheterogeneity in glycosylation patterns of α 1-AGP, α 1-ACT and Tf in sera of patients suffering from ulcerative colitis.

MATERIALS AND METHODS

Twenty-seven patients suffering from biopsy-proven ulcerative colitis and 17 healthy control subjects were studied.

The sera were separated by centrifugation after clotting and stored at -70°C. Rocket immunoelectrophoresis was used to determine the total serum concentrations of AGP, ACT, Tf, and CRP.

Microheterogeneity of ACT, AGP and Tf was analysed by crossed immunoaffinity electrophoresis (CIAE) with lectin-concanavalin A. The lectin-concanavalin A was

included in the first-dimension gel as the diantennary-specific affinocomponent. Separation of different glycoforms of proteins in sera was done *via* electrophoresis of these fluids with a concanavalin A-containing (Con A) polyacrylamide slab gel. Proteins lacking glycans of the diantennary type are not retarded by Con A, whereas proteins containing one or more diantennary glycans bind to Con A, and are electrophoretically retarded in the gel. Detection of the separated glycoforms was achieved through electrophoresis in the second dimension, using the polyclonal protein-specific anti-IgG antibody. The resulting precipitation lines were stained, and the relative occurrences of lectin-retarded and lectin-nonretarded glycoforms were calculated from the areas under the curves.

AGP, ACT and Tf in sera were separated into four variants: W 0-3 for AGP, A 1-4 for ACT, T 1-4 for Tf. Furthermore, the following parameters were measured: standard electrophoresis of serum proteins, iron (Fe) concentration, total iron binding capacity (TIBC), CRP, haemoglobin concentration and blood cell count.

RESULTS

Clinical characteristics and laboratory data

Twenty-seven patients suffering from ulcerative colitis were studied (16 females, 11 males). The patients were categorised according to the clinical activity index for ulcerative colitis using Trulove and Witts' classification of ulcerative colitis (severe $n = 9$, moderate $n = 10$ and mild $n = 8$). There were no significant differences in age, gender or body mass index (BMI) among the three studied groups (mean age 41.8 \pm 18 years, mean BIM 21.858 \pm 3.58 kg/m²). The nutritional status evaluated by physical characteristics was also comparable in the three groups (Table 1).

The haemoglobin concentration and platelet count were significantly lower in the severe group than in the mild group (11.17 \pm 1.92 g/dL and 13.11 \pm 2.38 g/dL, respectively, $P < 0.05$; and 428.78 \pm 116.65 thsd/mm³ and 257 \pm 173.18 thsd/mm³, respectively, $P < 0.05$). The serum iron concentration was significantly lower in the severe group than in the moderate (52.3 \pm 19.2 μ g/dL and 73.3 \pm 26.7 μ g/dL, respectively, $P < 0.05$) and mild groups (52.3 \pm 19.2 μ g/dL and 99 \pm 45.2 μ g/dL, respectively, $P < 0.05$). The white blood cell count was higher in the severe group than in the mild group (10.2 \pm 5.61 thsd/mm³ and 6.48 \pm 1.6 thsd/mm³, respectively, $P < 0.05$).

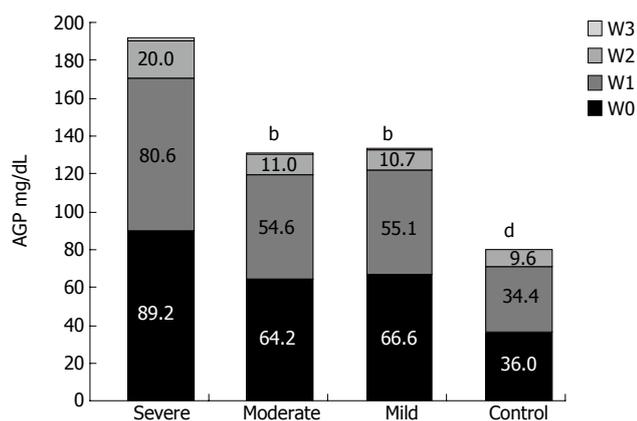


Figure 1 Microheterogeneity of AGP in sera of UC patients and healthy controls. ^b $P < 0.001$ vs severe UC patients; ^c $P < 0.001$ vs healthy subjects.

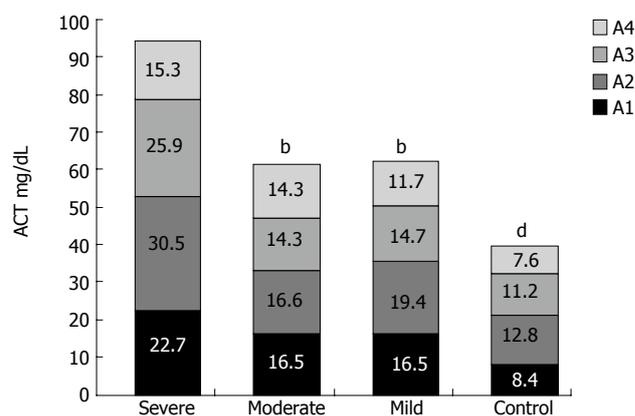


Figure 2 Microheterogeneity of ACT in sera of UC patients and healthy controls. ^b $P < 0.001$ vs severe UC patients; ^d $P < 0.001$ vs healthy subjects.

There were also significant differences in total protein concentration and albumin concentration between severe and moderate groups (5.72 ± 1.2 g/dL and 6.52 ± 0.71 g/dL, respectively, $P < 0.05$; 2.7 ± 0.78 g/dL and 3.63 ± 0.59 g/dL, respectively, $P < 0.05$).

Acute phase proteins

The CRP concentration was significantly higher in the severe group than in the moderate and mild groups (49 ± 34 mg/L and 5 ± 8.4 mg/L, respectively, $P < 0.001$; 49 ± 34 mg/L and 15 ± 24.42 mg/L, respectively, $P < 0.05$).

The serum concentration of AGP was significantly higher in all UC patients than in healthy subjects (151.3 ± 65.5 mg/dL and 80 ± 11 mg/dL, respectively, $P < 0.001$). However, there were also significant differences between the three groups. The highest concentration of AGP was found in the severe group (severe to moderate group: 191.1 ± 77.6 mg/dL and 130.3 ± 40.7 mg/dL, respectively, $P = 0.02$; severe to mild group: 191.1 ± 77.6 mg/dL and 132.9 ± 62.4 mg/dL, respectively, $P = 0.053$) (Figure 1).

The serum concentration of ACT was also elevated in all studied patients compared to that of the control group (72.8 ± 29.5 mg/dL and 40 ± 5.2 mg/dL, respectively, $P < 0.001$). Similar to AGP, the ACT concentration was significantly higher in the severe group than in the moderate and mild groups (severe to moderate group: 94.3 ± 36.7 mg/dL and 61.8 ± 18 mg/dL, respectively, $P = 0.01$; severe to mild group: 94.3 ± 36.7 mg/dL and 62.2 ± 19.2 mg/dL, respectively, $P = 0.02$) (Figure 2). There were no statistically significant differences in AGP and ACT serum concentrations between the moderate and mild groups.

As a negative acute phase protein, serum transferrin concentration was decreased in patients with ulcerative colitis compared to that in the healthy volunteers (237.0 ± 82.3 mg/dL and 352.0 ± 59.1 mg/dL, respectively, $P < 0.001$). There was no difference in the serum transferrin concentration between the three groups (Figure 3).

Microheterogeneity of acute phase proteins

The increased concentrations of AGP and ACT were not associated with a large shift in the microheterogeneity patterns.

Interestingly, we noticed a strong positive correlation

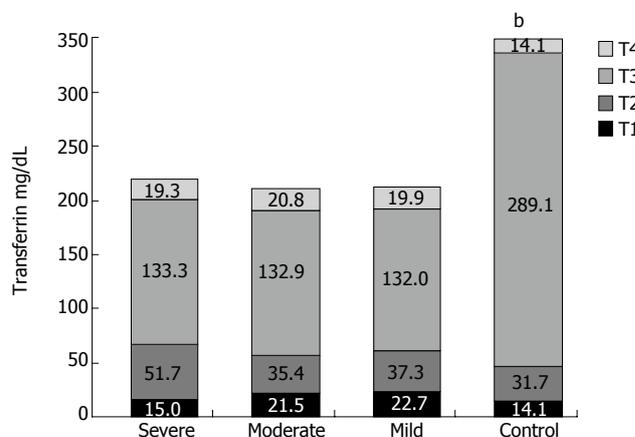


Figure 3 Microheterogeneity of Tf in sera of UC patients and healthy controls. ^b $P < 0.001$ vs healthy subjects.

between the serum AGP concentration, particularly W1 glycoform of AGP and platelet count ($r = 0.578$, $P = 0.0016$). The negative correlation was found between the albumin and serum AGP concentrations ($r = -0.448$, $P < 0.05$) as well as ACT concentration ($r = 0.429$, $P < 0.05$). A particular strong negative relationship occurred between W1 serum fraction of AGP ($r = 0.444$, $P = 0.0296$) as well as A2 serum fraction of ACT and albumin concentration ($r = 0.578$, $P = 0.003$). There was also a positive correlation between the platelet count and the serum concentration of A2 glycoform of ACT ($r = 0.39$, $P < 0.05$).

Decreased serum transferrin concentration in ulcerative colitis patients was accompanied with redirection of glycan synthesis to the highly branched and sialylated glycan. We noticed that the percentage of T2 (bi-, tri- and tetra-antennary) and T1 (tetra- and more antennary) fractions of transferrin were significantly higher in the investigated groups than in healthy subjects, while the percentage of T3 (biantennary) and T4 (asialylated, carbohydrate-deficient) fractions were lower.

We noticed that the correlation between T2 variant of transferrin and platelet count, as well as α_2 protein electrophoretic fraction was positive ($r = 0.432$, $P < 0.05$ and $r = 0.407$, $P < 0.05$). The negative correlation was found between this particular T2 variant and albumin

concentration ($r = 0.407$, $P < 0.05$).

The positive correlation between the serum concentration of T4 glycoform of transferrin and haemoglobin concentration was observed ($r = 0.405$, $P < 0.05$). We also found a negative correlation between T3 glycoform and AGP concentrations, as well as the α_1 electrophoretic fraction of proteins ($r = 0.48$, $P < 0.05$ and $r = 0.475$, $P < 0.05$).

DISCUSSION

Measurement of acute phase proteins may bring new insights into the mechanisms of inflammatory reactions occurring in ulcerative colitis. In our study, both during remission and exacerbation of ulcerative colitis the glycosylation profiles of AGP and ACT were altered. AGP variant W3 was not present in sera of both healthy individuals and ulcerative colitis patients during remission. However, it appeared in sera of the same patients during exacerbation. This variant is always present in the sera of patients after inflammation, infection, burn or other severe tissue damage. Its presence in the sera of our studied patients was probably caused by the active necrotizing and inflammatory processes in the gut. The high concentration of ACT demonstrated in our patients, is also characteristic for disorders where tissue necrosis occurs (e.g. in burns).

Though the clinical image can confirm ulcerative colitis (UC) is a chronic inflammatory disease, alternations in APP concentrations and microheterogeneity depict rather a domination of "acute inflammatory image" that is probably caused by IL-6. Even in remission of UC the glycosylation profile of the best described AGP does not return to normal but remains altered, with a relative increase of Con A reactive (thus: biantennary) glycoforms. This constant inflammatory stimulation probably contributes to altered Tf glycosylation and, as a consequence to deteriorated iron metabolism and anaemia. In our study, the concentration of transferrin, a negative acute phase protein, was markedly decreased in the acute phase of ulcerative colitis, suggesting that low concentration and especially changes of the glycosylation profile of Tf may be responsible for the impaired iron metabolism in patients suffering from ulcerative colitis.

In sera of healthy individuals, transferrin separates into four variants: T1 weakly reacting with Con A (with mainly triantennary glycans), T2 (with one biantennary glycan and one triantennary glycan), T3 (with two biantennary glycans) and T4 (with probably defective sugars). The variant T3 covers about 80% of the total protein and is the main fraction able to bind to iron, consisting mainly of apo-transferrin. Our study showed the alterations of Tf glycosylation in sera of patients suffering from ulcerative colitis. It is well known that existing anaemia in UC patients is not only to the blood loss with a stool, but also due to the chronic inflammatory process. It is possible that the different glycoforms of Tf have a different iron affinity that may affect the iron balance in the organism.

A correlation was shown between the concentration of transferrin T2 variant, bearing partially triantennary glycans and platelet count as well as α_2 protein electrophoretic fraction, whereas a negative correlation was shown

between T2 variant and albumin concentration. We are thus able to show that inflammation influences the microheterogeneity of transferrin. The higher intensity of the inflammatory process is combined with deteriorated iron transport ability to transferrin, which may intensify anaemia.

The present study revealed a strong negative correlation between variant T1 of transferrin, α_1 -acid glycoprotein and α_1 -antichymotrypsin concentration. A negative correlation was observed between the patient status and iron concentration. Disturbances in the iron balance (low TIBC and iron concentration) reflected a higher amount of variant T1 of transferrin in the sera of UC patients.

In conclusion, glycosylation patterns of transferrin in ulcerative colitis patients shift to the highly branched and sialylated glycans compared with those in healthy subjects. However, the glycosylation patterns of transferrin do not differ according to the activity index of ulcerative colitis. The microheterogeneity patterns of AGP and ACT are similar in ulcerative colitis patients and healthy subjects, though the total concentrations of these acute phase proteins are increased due to the disease.

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RAPID COMMUNICATION

Smoking is not associated with nonalcoholic fatty liver disease

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Abstract

AIM: To analyze the relationship between smoking and nonalcoholic fatty liver disease (NAFLD).

METHODS: This is a cross-sectional study of a healthy population, carried out in a check-up unit of a university hospital in Mexico City. We enrolled 933 subjects, 368 current smokers (cases) and 565 persons who had never smoked (controls). Demographic, metabolic and biochemical variables were measured in the two groups. NAFLD was determined by ultrasound and metabolic syndrome according to ATPIII.

RESULTS: A total of 548 men (205 cases and 343 controls) and 337 women (114 cases and 223 controls) were included in the analysis. Statistical differences between cases and controls were observed only in high blood pressure prevalence (6.6% vs 11.3%, $P < 0.05$; cases and controls respectively), high-density lipoproteins (1.00 ± 0.26 vs 1.06 ± 0.28 mmol/L, $P < 0.005$), triglycerides (2.18 ± 1.49 vs 1.84 ± 1.1 mmol/L, $P < 0.001$), and erythrocyte sedimentation rate (11.3 ± 9.3 vs 13.5 ± 11.9 mm/h, $P < 0.001$). No differences were observed in the prevalence of NAFLD (22.27% vs 29.68%, $P = \text{NS}$) and metabolic syndrome (41.69% vs 36.74%, $P = \text{NS}$). Univariate analysis showed that smoking was not a risk factor for NAFLD (OR = 0.89, 95% CI 0.65-1.21).

CONCLUSION: No differences in NAFLD prevalence were observed between current smokers and nonsmokers, and furthermore, no differences were observed in heavy smokers (more than 20 packs/year), indicating that there is no relationship between smoking and NAFLD.

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Key words: Smoking; Nonalcoholic fatty liver disease; Metabolic syndrome

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is an increasingly recognized condition that may progress to end-stage liver disease^[1]. The clinical implications of NAFLD are derived mainly from its common occurrence among the general population (from 2.8% to 25%) and its potential to progress to fibrosis, cirrhosis and liver failure^[2].

Obesity or a high body mass index (BMI) is a major risk factor for development of liver disease, and the prevalence of NAFLD is elevated 4.6-fold among obese people. In obese subjects, metabolic syndrome that links type 2 diabetes mellitus, dyslipidemia and hypertension to a visceral or male pattern of adipose tissue distribution is a more important risk factor for most comorbidities of obesity than obesity *per se*^[3]. Other risk factors associated with NAFLD are waist circumference (> 102 cm for males and > 88 cm for females)^[4], hyperinsulinemia, hypertriglyceridemia and impaired glucose tolerance or type 2 diabetes^[5,6]. In fact, hepatic steatosis (HS) is now considered to be the hepatic manifestation of insulin resistance (IR)^[7].

Smoking is a well-studied risk factor for many malignant neoplasms, cardiovascular diseases, chronic obstructive pulmonary diseases and other important diseases^[8]. However, it was recently shown that smoking is associated with many of the risk factors for NAFLD, particularly obesity^[9], IR, diabetes and dyslipidemia^[10].

Basic and clinical research demonstrates that smoking alters enzymatic and inflammatory pathways in liver physiology^[11,12], and is considered to be a risk factor for liver neoplasm, and affects the prognosis of chronic liver diseases^[13,14].

Currently, there is no information available about the epidemiologic importance of smoking in NAFLD. The aim of this study was thus to investigate the relationship between smoking and NAFLD.

MATERIALS AND METHODS

Study population

We conducted a cross-sectional study in the Check-up

Unit of the Diagnostic Clinic at the Medica Sur Clinic and Foundation (a university hospital with subspecialty care) in Mexico City, Mexico. This hospital provides private care for mainly middle- and high-income individuals from Mexico City and the surrounding metropolitan areas. Our sample population was formed from a series of consecutive asymptomatic subjects who were referred to the Check-up Unit by their companies as an annual requirement. The study was approved by the Human Subjects Committee of The Medica Sur Clinic and Foundation as conforming to the ethical guidelines of the 1975 Declaration of Helsinki, and written informed consent was obtained from all participants before entry. After informed consent was obtained, all patients were asked to complete a questionnaire that included demographic and medical variables. Cases were defined as current tobacco smokers (and were classified according to the number of packets smoked per year), while controls were defined as patients who had never smoked.

Questionnaire

Subjects were asked to complete a questionnaire that asked for information on demographic data, age, gender, alcohol consumption, smoking habits, diabetes mellitus, hypertension, chronic liver disease, hyperlipidemia, and the use of drugs that predispose to NAFLD.

Physical examination

Body weight was measured, in light clothing and without shoes, to the nearest 0.10 kg. Height was measured to the nearest 0.5 cm. BMI was calculated as weight (kg) divided by height (m) squared. Waist circumference (to the nearest 0.1 cm) was measured at the midpoint between the lower border of the rib cage and the iliac crest, and hip circumference was similarly obtained at the widest point between hip and buttock.

Three blood pressure readings were obtained at 1 min intervals, and the second and third systolic and diastolic pressure readings were averaged and used in the analyses.

Smoking exposure

In a detailed questionnaire, all participants were asked about smoking frequency, duration and type of cigarette. Secondhand smoking was not evaluated. Subjects exposed to chemical or industrial gases were excluded, as were those regularly exposed to other environmental smoke (i.e., open fires using firewood and crop residues as fuel). Based on the number of cigarettes and years of smoking, we calculated pack/year (each pack was considered consisting of 200 cigarettes).

Metabolic syndrome

Participants having three or more of the following criteria were defined as having the metabolic syndrome. The criteria were defined according to the Executive Summary of the Third Report of the National Cholesterol Education Program^[15]. (1) Abdominal obesity: Waist circumference > 102 cm in men and > 88 cm in women; (2) Hypertriglyceridemia: Triglycerides \geq 1500 mg/L (1.69 mmol/L); (3) Low high-density lipoprotein (HDL)

cholesterol: HDL < 400 mg/L (1.04 mmol/L) in men and < 500 mg/L (1.29 mmol/L) in women; (4) High blood pressure: \geq 130/85 mmHg (17.33/11.33 kPa); (5) High fasting glucose: \geq 1100 mg/L (\geq 6.1 mmol/L).

Hepatic steatosis

The diagnosis of NAFLD was first suspected in those subjects with a record of at least six months of abnormal serum aminotransferase levels that were not related to other causes of liver disease, including hepatitis B and C virus infections, autoimmune disorders, alcohol consumption or hemochromatosis, and who had ultrasonographic findings compatible with HS. Real-time ultrasonographic studies were performed while the subjects were fasting. A 3.5 MHz transducer (Elegra; Siemens Medical Systems, Mountain Grove, CA, USA) was used to obtain the following images: sagittal view of the right lobe of the liver and right kidney; transverse view of the left lateral segment of the liver and spleen; transverse view of the liver and pancreas, and any focal areas of altered echotexture. The protocol used to evaluate the pattern of HS by ultrasound was graded according to the method of Saadeh *et al.*^[16]. In the second evaluation, all studies for each subject were viewed side-by-side in a masked fashion ($\kappa = 0.92$).

Analytical procedures

Plasma glucose of subjects in the fasting state was measured in duplicate using an automated analyzer. The coefficient of variation for a single determination was 1.5%. Cholesterol, HDL-cholesterol and triglyceride concentrations were measured by enzymatic colorimetric methods, using CHOL, HDL-C plus (second generation) and triglyceride assays (Roche Diagnostics Co., Indianapolis, IN, USA), respectively. Low-density lipoprotein (LDL) cholesterol concentrations were calculated using the Friedewald formula^[17].

Statistical analyses

Means and standard deviations were used to describe the distributions of continuous variables in comparisons between smokers and controls. The nonparametric Mann-Whitney *U* test was applied because of the nonnormal distribution of some of these variables. By means of cross-tabulations, the risks associated with the probability of having HS were estimated. Odds ratios (ORs) were calculated with the independent variables coded in a binary form. Statistical significance was determined by Fisher's exact test (two-tailed) and 95% confidence intervals. To derive adjusted OR (by confounders) associated with the probability of HS, multivariate unconditional logistic regression analyses were conducted. Multicollinearity in the adjusted models was tested by deriving the covariance matrix. All statistical analyses were conducted using the statistics program SPSS/PC version 12.0 (Chicago, IL, USA).

RESULTS

We enrolled 885 consecutive subjects: 319 current smokers

Table 1 Comparative characteristics between smokers and nonsmokers

Variable	Smokers (n = 319) (mean ± SD)	Non-smokers (n = 566) (mean ± SD)	P
Age (yr)	46.9 ± 11.19	46.8 ± 12.05	NS
Weight (kg)	75.5 ± 15.99	73.84 ± 14.14	NS
Gender n (%)			
Male	205 (64.26)	343 (60.60)	NS
Female	114 (35.74)	223 (38.40)	
Height (m)	1.68 ± 0.09	1.66 ± 0.09	NS
BMI (kg/m ²)	26.8 ± 4.49	26.6 ± 4.02	NS
Overweight and obesity n (%)			
BMI ≥ 25 kg/m ²	205 (64.26)	369 (65.19)	NS
BMI ≥ 30 kg/m ²	54 (16.93)	105 (18.55)	NS
Waist circumference (cm)	84.3 (11.2)	83.4(11.5)	NS
Glucose (mmol/L)	5.42 ± 1.53	5.42 ± 1.52	NS
Type 2 diabetes mellitus n (%)	12 (3.7)	13 (2.3)	NS
High blood pressure (> 17.33/11.33 kPa) n (%)	21 (6.6)	64 (11.3)	< 0.05
Cholesterol (mmol/L)	5.4 ± 1.14	5.33 ± 0.95	NS
C-LDL (mmol/L)	3.48 ± 0.97	3.46 ± 0.85	NS
C-HDL (mmol/L)	1.0 ± 0.26	1.06 ± 0.28	< 0.005
Triglycerides (mmol/L)	2.18 ± 1.49	1.84 ± 1.10	< 0.001
C-reactive protein (mg/L)	3.5 ± 6.03	3.4 ± 5.10	NS
Erythrocyte sedimentation rate (mm/h)	11.3 ± 9.38	13.5 ± 11.90	< 0.001
Albumin (g/L)	4.0 ± 0.28	4.0 ± 0.31	NS
Total bilirubin (mg/L)	0.9 ± 0.39	1.27 ± 8.31	NS
ALT (UI/L)	31.4 ± 20.33	33.3 ± 26.8	NS
AST (UI/L)	26.9 ± 10.25	28.9 ± 16.28	< 0.05
Metabolic syndrome n (%)	133 (41.69)	208 (36.74)	NS
ALT 2 times upper normal limit value n (%)	4 (1.25)	8 (1.41)	NS
Hepatic steatosis n (%)	87 (27.27)	168 (29.68)	NS

(205 men and 114 women) and 566 controls (343 men and 223 women). The general characteristics between groups are described in Table 1. We observed that both groups were very similar, although there were significantly more hypertensive subjects in the nonsmoker group (11.3% *vs* 6.6%, *P* < 0.05). In the current smokers group, serum levels of triglycerides were higher (2.18 ± 1.49 *vs* 1.84 ± 1.1 mmol/L, *P* < 0.005); While in contrast, they showed lower levels of HDL cholesterol (10.0 ± 206 *vs* 10.6 ± 2.8 mg/L, *P* < 0.001). Other differences between the groups were observed in erythrocyte sedimentation rate and AST values. We did not observe any differences in the prevalence of metabolic syndrome and HS. When only subjects with HS were analyzed according to smoking status, only differences in globulin values were significant (Table 2).

Univariate unconditional logistic regression analysis revealed no increased risk for HS in smoking subjects. Several models were tested, with smokers divided into subgroups depending on their smoking amount: > 10 packs/year but ≤ 20 packs/year, and > 20 packs/year, and then compared with all other subjects and with nonsmoking subjects only (Table 3). Although there appeared to be a dose-dependent effect, this could not be confirmed. Similar analyses were made using ALT values (twice the upper normal limit value) comparing

Table 2 Comparative characteristics in liver function tests between smokers and nonsmokers (including only subjects with hepatic steatosis)

Variable	Smokers (n = 87) (mean ± SD)	Non-smokers (n = 168) (mean ± SD)	P
Albumin (g/L)	4.07 ± 0.23	4.10 ± (0.37)	NS
Total bilirubin (mg/L)	0.94 ± 0.31	0.93 ± 0.37	NS
ALT (UI/L)	43.7 ± 24.6	49.07 ± 40.7	NS
AST (UI/L)	32.3 ± 13.0	35.2 ± 22.9	NS
Alkaline phosphatase (UI/L)	71.2 ± 18.0	74.7 ± 24.0	NS
Lactate dehydrogenase (UI/L)	154.9 ± 24	160.6 ± 30	NS
ALT 2 times upper normal limit value n (%)	2 (2.4)	8 (4.8)	NS

Table 3 Univariate model for increased risk of hepatic steatosis

Variable	OR (95% CI)
Smoking (n = 319)	0.89 (0.65-1.21)
Smoking > 10 pack/yr (n = 152) ¹	1.30 (0.88-1.92)
Smoking > 10 pack/yr (n = 137) ²	1.16 (0.76-1.64)
Smoking > 20 pack/yr (n = 77) ¹	1.42 (0.84-2.43)
Smoking > 20 pack/yr (n = 64) ²	1.54 (0.94-2.52)

¹ *vs* smokers less than 10 packs/yr and nonsmokers; ² *vs* nonsmokers only.

Table 4 Univariate model for increased risk of ALT twice the upper normal limit value

Variable	OR (95% CI)
Smoking (n = 319)	0.88 (0.26-2.96)
Smoking > 10 pack/yr (n = 152) ¹	Not calculated ³
Smoking > 10 pack/yr (n = 137) ²	0.43 (0.05-3.39)
Smoking > 20 pack/yr (n = 77) ¹	Not calculated ³
Smoking > 20 pack/yr (n = 64) ²	Not calculated ³

¹ *vs* smokers less than 10 packs/yr and non-smokers; ² *vs* nonsmokers only; ³ Could not be calculated as there were no subjects with an ALT level twice the upper normal limit value in the specified group.

smoking and smoking intensity, which found no statistical associations (Table 4).

DISCUSSION

The liver is a complex organ that has numerous enzymatic pathways involved in its physiology; Many drugs can alter these delicate processes. In particular, substances derived from smoking could impair antioxidant mechanisms^[11], induce cytochrome P450^[18], other enzymatic pathways^[19,20] and inflammatory cytokines^[12]. Although the clinical importance of smoking in hepatology has been described, specifically in hepatocellular carcinoma^[13], chronic infection due to hepatitis C virus^[13,21] and alcoholic cirrhosis^[22], less is known about NAFLD. In the present study, we found that smoking was not associated with NAFLD or elevated liver enzymes in subjects without

chronic liver disease. Recently, a prospective study by Suzuki *et al.*^[23] demonstrated the importance of smoking in increased levels of ALT (only in subjects who started smoking in the study); This finding supported a putative relationship between NAFLD and smoking, although in our study, the effect of chronic smoking did not appear to be significant. Indeed, such an association is implicated by data other than an epidemiological study. Currently, smoking is known to be associated with several metabolic disturbances that are considered risk factors for NAFLD. In large study cohorts, smoking increases the prevalence of diabetes mellitus, and subjects who smoke have greater IR^[24], which are considered to be the hallmarks of NAFLD^[2,25].

In this study, we did not find associations between smoking and smoking intensity (number of packs/year), and the prevalence of HS and ALT at twice the upper normal limit value. As the data showed both samples were very similar. It suggests that smoking does not affect directly the prevalence of NAFLD, despite our inability to evaluate insulin values and the IR index.

Another important risk factor for NAFLD is obesity, and especially the distribution of adipose tissue. The study of den Tonkelaar *et al.*^[9] clearly showed that smoking could influence the distribution of adipose tissue, with an increased waist/hip ratio that is considered metabolically important^[26], in other nonrelated liver diseases^[27-29] and NAFLD^[30]. In this study, we did not observe statistical differences related to obesity, fasting serum glucose levels or prevalence of metabolic syndrome; We found minimal (but significant) differences between current smokers and nonsmokers in levels of serum triglycerides and HDLs. This indicates that the samples are very similar, and the primary variable of the study (smoking) does not influence the prevalence of NAFLD. We observed that smoking subjects had a lower prevalence of high blood pressure, where this unexpected result did not change the results in the multivariate analysis (data not shown). Similar findings are reported in samples that analyze metabolic syndrome in non-diabetic subjects^[31,32]. This phenomena could be due to a long term exposure to nicotine that alters stress response resulting in a reduction in the number or affinity of receptors mediating effects of nicotine in different central nervous system structures that integrate the neuroendocrine stress response and may also lead to lower responses of other stress hormones (ACTH, prolactin, growth hormone) to a variety of stimuli^[33].

However, limitations of the study should be mentioned, which could explain the lack of association between smoking and NAFLD. First, we did not evaluate IR; the information derived from this variable could partially explain our results and clarify whether smoking impairs insulin sensitivity in our population (particularly considering a potential bias because our sample includes only a selected group of subjects). The other factor that helps to explain this result is that we did not evaluate physical activity, which is another factor related to IR and possibly affected by smoking^[34]; Ultrasound is non-invasive but highly unspecific in the diagnosis of NAFLD, but in large populations it does not confer ethical conflicts.

Finally the low number of subjects with ALT values twice the upper normal limit value, could underpower statistical models.

In conclusion, this is the first study that has analyzed specifically the importance of smoking in NAFLD. Although some evidence suggests a role in this disease, we could not observe differences between current smokers and nonsmokers; Furthermore, no differences are found in heavy smokers (> 20 packs/year), indicating that there is no relationship between smoking and NAFLD.

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On attitudes about colorectal cancer screening among gastrointestinal specialists and general practitioners in the Netherlands

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Abstract

AIM: To find out whether there are differences in attitudes about colorectal cancer (CRC) screening among gastrointestinal (GI) specialists and general practitioners (GPs) and which method is preferred in a national screening program

METHODS: Four hundred and twenty Dutch GI specialists in the Netherlands and 400 GPs in Amsterdam were questioned in 2004. Questions included demographics, affiliation, attitude towards screening both for the general population and themselves, methods of screening, family history and individual risk.

RESULTS: Eighty-four percent of the GI specialists returned the questionnaire in comparison to 32% of the GPs ($P < 0.001$). Among the GI specialists, 92% favoured population screening whereas 51% of GPs supported population screening ($P < 0.001$). Of the GI specialists 95% planned to be screened themselves, while 30% of GPs intended to do so ($P < 0.001$). Regarding the general population, 72% of the GI specialists preferred colonoscopy as the screening method compared to 27% of the GPs ($P < 0.001$). The method preferred for personal screening was colonoscopy in 97% of the GI specialists, while 29% of the GPs favoured colonoscopy ($P < 0.001$).

CONCLUSION: Screening for CRC is strongly supported by Dutch GI specialists and less by GPs. The major health issue is possibly misjudged by GPs. Since GPs play a crucial role in a successful national screening program, CRC awareness should be realized by increasing knowledge about the incidence and mortality, thus increasing awareness of the need for screening among GPs.

INTRODUCTION

Colorectal cancer (CRC) is the second leading cause of cancer-related death in the Western world, with over 4400 deaths per year in The Netherlands and 500 000 deaths per year worldwide^[1]. Due to the aging population and population growth, the expected number of CRC patients will increase in the forthcoming years^[2]. CRC is suitable for screening. Pre-malignant lesions can be identified and removed, and in case of detection in an early stage, its five year survival rate exceeds 90%^[3].

The call for a national screening program in the Netherlands is increasing and implementation of the faecal occult blood test (FOBT) as a method of screening is planned within 2-3 years^[4]. A meta-analysis of results from four randomised controlled trials showed that screening reduces mortality of colorectal cancer patients by 23% of those who have been actually screened^[5]. However, a recent study has shown a moderate sensitivity (12%) in detecting CRC with FOBT and patient compliance of only 40%-60%^[6]. Mortality reduction by means of endoscopic screening is expected to be 15%-20% higher, but evidence from prospective randomized trials is lacking^[7].

The Netherlands rank the lowest public awareness regarding CRC among countries in Europe^[8]. In some countries feasibility and implementation studies of nationwide screening programs, together with capacity inventories, are ongoing, while Dutch reports are still awaited^[9-11].

A high participation level of the general population is essential for a national screening program to succeed. The key to achieving adequate compliance is informing the community through an educational campaign about the nature and extent of the disease, as well as education re-

Table 1 Population and personal screening

	GI specialists (%)	GPs (%)	Fisher exact test
In favour of population screening	92	51	$P < 0.001$
In favour of personal screening	95	30	$P < 0.001$

Table 2 Favoured method of population screening

	GI specialists (%)	GPs (%)	Fisher exact test
FOBT	0	26	$P < 0.001$
Sigmoidoscopy	0	18	$P < 0.001$
Combination FOBT + sigmoidoscopy	12	0	$P < 0.001$
Colonoscopy	72	27	$P < 0.001$
Fecal DNA test/CT-colonoscopy	16	19	NS ($P = 0.49$)
Indifferent/Unknown	0	10	$P < 0.001$

garding the method of screening to be used. Involvement of the general practitioners (GPs) is crucial in increasing the yield of a CRC screening program with FOBT^[12-14].

There is ongoing debate among physicians and politicians on the necessity of CRC screening and which screening modality is to be used. This study was to inquire into the attitudes regarding screening among Dutch gastroenterologists, gastrointestinal (GI) surgeons and GPs.

MATERIALS AND METHODS

During a biannual meeting in 2004 in the Netherlands, addressing all general topics in gastroenterology, all the attending Dutch gastroenterologists and gastrointestinal surgeons were questioned ($n = 420$). The same 17-item questionnaire was sent to all GPs in Amsterdam ($n = 400$) in fall, 2004. In an attempt to achieve a comparable sample size between GI specialists and GPs, only GPs from Amsterdam were asked to return the questionnaire. Questions included demographics, affiliation, attitude towards screening both for the general population and themselves, methods of screening, family history and individual risk.

Statistical analysis

SPSS for Windows version 11.0 was used for the descriptive statistical analysis. The Fisher exact test was used for the comparison of proportions. $P < 0.05$ was considered statistically significant.

RESULTS

Responses were obtained from 354 of the 420 GI specialists (84%) who were questioned. Among the GI specialists 82% were gastroenterologists and 18% gastrointestinal surgeons. Eleven percent of the GI specialists worked in an academic setting and 89% in a community hospital. The mean age of GI specialists was 48

Table 3 Age at initiation of screening

	GI specialists (%)	GPs (%)	Fisher exact test
50 yr	37	29	NS ($P = 0.39$)
55 yr	42	18	$P = 0.005$
60 yr	21	13	NS ($P = 0.29$)
Unknown	0	40	$P < 0.001$

Table 4 Familial predisposition to CRC and personal judgment of increased risk of developing CRC

	GI specialist (%)	GPs (%)	Fisher exact test
Familial predisposition for CRC ¹	4	25	$P < 0.001$
Personal judgment of increased risk for CRC	2	8	$P = 0.006$

¹ Defined as one or more first degree relatives diagnosed with CRC.

years (range 28-71 years). The response rate among the 400 potentially eligible GPs was 32% (126). All GPs worked in Amsterdam. The mean age of the GPs was 49 years (range 32-69 years).

A highly significant difference in appreciation of population and personal screening was found between GI specialists and GPs ($P < 0.001$, Table 1). Moreover, significant differences were found in the preferred screening method. Colonoscopy was considered the primary population screening tool by the majority of GI specialists, while FOBT, sigmoidoscopy and colonoscopy were almost equally supported by GPs (Table 2). Personal screening with colonoscopy was favoured by 97% and 27% of GI specialists and GPs, respectively ($P < 0.001$). Forty-two percent of GI specialists considered 55 years as the proper age to start personal screening, whereas the age of 50 years was chosen by GPs (Table 3). Four percent of GI specialists and 25% of GPs reported a familial predisposition to CRC. Nevertheless, within the latter two groups only 50% and 33% regarded themselves at a higher risk of developing CRC (Table 4). Finally, a subgroup analysis was performed on the GPs with familial predisposition to CRC, regarding their opinion on population screening. In this group 61% preferred population screening, compared to 51% in the total group of GPs ($P = 0.32$).

DISCUSSION

Population screening for CRC is strongly supported by Dutch GI specialists. In their opinion benefits definitely outweigh the drawbacks and their discussion focuses on how to implement a national CRC screening program and which method should be used. Unfortunately, the above-mentioned results suggest that GPs are more reluctant to speak out in favour of a CRC population-based screening program than GI specialists. Only 51% of responding GPs are in favour of population screening. A challenging task is reserved for the GI specialists to convince GPs of the need for screening. It has been shown that by increasing

knowledge about the incidence and mortality of CRC, as well as the possibility for early detection, the attitudes of GPs will change^[15].

There is a remarkable difference in returned questionnaires between GI specialists and GPs. It can be hypothesized that completing a short questionnaire on a meeting is less of a burden than during daily routines, where other priorities may prevail. However, the response rates differ significantly, suggesting that GPs are ignorant of the CRC screening issue or are in a low state of awareness of the problem's magnitude. These findings correspond to the previous reports stating a lack of interest and knowledge in the definition of the high risk population among GPs^[16,17].

Another argument may be that only half of all GPs favour population screening and only 30% intend to be screened themselves. In the present study, no significant difference was observed in preference of population screening (61% *vs* 51%; $P = 0.32$) even between a subgroup of GPs with a familial predisposition to CRC which results in an increased risk of developing CRC and the total group of GPs. In contrast, 92% of all the GI specialists supported such a nation-wide CRC screening program and 95% planned to undergo personal screening. In this context, it can be put forward that knowledge of the natural course of CRC, its pre-malignant precursor lesions and therapeutic options in case of early detection, might explain the large differences in opinion. Since GPs play a crucial role in achieving a successful national screening program, the latter suggestion should be a concern to GI specialists and central government. On the other hand, GPs are subjected to a continuous barrage about the different types of screening (lung, breast, prostate, CRC, cervix) and they might have a wider view on priorities and cost-efficiency in the health sector.

Regarding the screening modalities for both personal and population screening, a clear preference to colonoscopy was observed among GI specialists.

Colonoscopy is the method of screening preferred by GI specialists. In the present study, 97% of the GI specialists preferred this method for their personal screening, and 72% for population screening. The higher preference to alternative methods for population screening presumably reflects concerns regarding the capacity and logistics (Table 2).

Among GPs, population screening with FOBT or colonoscopy was equally supported (respectively 26% and 27%). A rather surprising finding is that none of the GI specialists preferred FOBT as a screening method, since there is evidence in terms of cost-effectiveness and mortality reduction^[18-20]. Nevertheless, the fact that GI specialists agree with and encourage the implementation of FOBT for nationwide screening can be explained by the fact that FOBT is the only screening method accepted by the central government at this moment. Furthermore, there is a convincing mortality reduction using FOBT as a method of screening and in this perspective screening with FOBT is better in any case than no screening^[5,18]. In addition, an ongoing screening program allows future alternatives to be implemented more easily, when they are proven superior to FOBT.

Finally, a large proportion of GI specialists plan to start

personal screening at the age of 50-55 years, even though only 4% are found at a high risk of developing CRC. In 40% of responding GPs in favour of personal screening, the age at which screening should be initiated is unknown (Tables 3 and 4). This emphasises the importance of education about this disease since one out of 20 people will develop CRC during a lifetime, with advancing years of age as the foremost risk factor for CRC development. The highest mortality rate for CRC appears to be around the age of 60 years, the time interval in which a precursor lesion develops into an invasive cancer is 10-15 years^[21].

In conclusion, the findings of our study are relevant to GPs in Amsterdam. However it is unknown whether GPs in other parts of the Netherlands have similar attitudes. Nonetheless, this inquiry clearly indicates the urgent need for GI specialists and GPs to solve the ongoing debate on CRC screening. Education of all parties involved should lead to an increased knowledge about the magnitude of the CRC problem. An unambiguous policy stressing the crucial role of GPs in a CRC screening program, may improve patient compliance, thereby reducing the mortality of CRC. More studies are mandatory to draw firm conclusions.

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Association of *H pylori cagA* and *vacA* genotypes and IL-8 gene polymorphisms with clinical outcome of infection in Iranian patients with gastrointestinal diseases

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10.47; $P = 0.005$).

CONCLUSION: The IL-8 -251 A/T polymorphism and the polymorphisms in *H pylori vacA* gene are involved in limiting the infection outcome to gastritis and peptic ulcer or in favoring cancer onset in Iranian patients.

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Key words: Interleukin 8; *H pylori*; Gastric cancer; Peptic ulcer; Polymorphism

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Abstract

AIM: To find out if a functional promoter polymorphism in the IL-8 gene along with *cagA* status and polymorphisms in *vacA* gene influence the type of diseases in Iranian patients infected by *H pylori*.

METHODS: IL-8 -251 A/T polymorphism was genotyped by oligonucleotide allele specific PCR (ASO-PCR) in a sample of 233 patients with *H pylori* infection undergoing upper gastrointestinal endoscopy. The presence of *cagA* gene and polymorphisms in *vacA* gene was also determined by PCR. Association of these genetic polymorphisms with the development of gastritis, peptic ulcers as well as gastric cancer was tested.

RESULTS: When the patients with different clinical manifestations were compared according to the presence of *cagA* gene or various *vacA* genotypes, only the *vacA* genotypes were significantly different among gastritis, peptic ulcer and gastric cancer patients ($\chi^2 = 17.8$; $P = 0.001$). Furthermore, there was a significant difference in the frequency of IL-8 -251 A/T genotypes between patients with gastric cancer and benign diseases ($\chi^2 =$

INTRODUCTION

Infection with *H pylori* has plausible associations with a variety of clinical outcomes, including chronic gastritis, peptic ulcer and gastric cancer^[1-4]. Variation in the clinical outcome of *H pylori* induced pathology is multifactorial, involving a complex interplay between the host immune responses, pathogen virulence factors, and niche characteristics. Many putative virulence factors have been identified in *H pylori* that contribute to its pathogenesis. The 128-kDa cytotoxin-associated gene encoded antigen A (*cagA*)^[5] and vacuolating cytotoxin antigen gene (*vacA*)^[6] are known as the most important ones. *cagA* gene was identified as a strain-specific *H pylori* gene and has been recognized as a marker for strains that confer increased risk for peptic ulcer disease^[7-8] and gastric cancer^[9]. The *cagA* gene is present downstream of a 40-kb cluster of virulence genes known as the *cag* pathogenicity island (*cag*-PAI). These genes encode a type IV secretion system that forms a syringe-like structure to translocate the immunodominant *cagA* protein into the gastric epithelial cells. *cag*-PAI has also been implicated in the induction of IL-8 in cultured gastric cells^[10]. This property contributes to the proinflammatory power of the strains and thus

to their virulence capability. The difference between *H pylori* strains in virulence capability is also dependent on the expression of *vacA* (87 kDa), which is toxic to epithelial cells *in vitro*^[11-13]. Moreover, mice which were administered *vacA* orally developed gastric ulcers^[14]. Unlike the *cagA*, *vacA* gene is conserved among all *H pylori* strains, although significant polymorphism exists in its gene^[15]. *vacA* alleles possess one of two types of signal regions, s1 or s2, and one of two mid-regions, m1 or m2, occurring in all possible combinations. The *vacA* signal region encodes the signal peptide and the N-terminus of the processed *vacA* toxin: type s1 *vacA* is fully active, but type 2 has a short N-terminus extension that blocks vacuole formation^[16]. *vacA* s2 strains are rarely isolated from patients with peptic ulcers or gastric adenocarcinoma^[15]. The *vacA* mid-region encodes part of the toxin cell binding domain. Vacuolating activity is higher in s1/m1 genotypes than in s1/m2 genotypes, and is absent in s2/m2 genotypes^[16]. Consequently, *vacA* s1/m1 genotypes are more frequently associated with peptic ulceration and gastric carcinoma^[17,18]. The genetic heterogeneity in immune responses among individuals is another important factor which determines the clinical outcome of *H pylori* infection. Support for this consideration is provided by the low frequency of gastric cancer in some developing countries in spite of the paradoxically high prevalence of *H pylori* infection in those countries^[19,20]. Up to now, there are several reports indicating the association of IL-1 β , tumor necrosis factor α (TNF- α), and IL-10 gene polymorphisms with an increased risk of developing gastric atrophy, hypochlorhydria, and non-cardia gastric cancer^[21-23]. Due to the roles which are played by IL-8 in the pathogenesis of *H pylori* infection, the IL-8 gene is one of the most important candidate host genes in determination of the outcome of *H pylori* infection. This cytokine is produced by gastric epithelial cells as an early response to *H pylori* virulence factors, such as *cagA*^[10]. IL-8 is also a major host mediator involved in neutrophil and phagocyte chemotaxis and activation^[24,25], thereby causing mucosal damage by releasing reactive oxygen radicals^[25]. It is therefore tempting to speculate that mucosal IL-8 production due to *H pylori* infection may be an important factor in the immunopathogenesis of peptic ulcer diseases and may also be relevant in gastric carcinogenesis^[26]. Interestingly, previous studies have suggested that the production of IL-8 is genetically determined and neutrophils from individuals who are homozygous for the AA genotype at the -251 position demonstrated a trend toward higher levels of IL-8 production in response to lipopolysaccharides than those without the allele^[27]. More recently, Ohyauchi *et al* reported that IL-8 -251 A/T polymorphism influences the susceptibility of *H pylori* related gastric diseases in the Japanese population^[28]. Furthermore, in an *H pylori* infected Chinese population the risk of gastric cancer was also significantly elevated in patients with the IL-8-251 AA genotype^[29]. Considering the above information, the aim of this study was to look for an association between IL-8 -251 A/T polymorphism, *vacA* genotypes, the presence of *cagA* gene and clinical outcome of *H pylori* infection in Iranian patients.

MATERIALS AND METHODS

Patients and Bacterial strains

In the present study 298 patients were classified at the time of endoscopy into those having gastritis ($n = 199$), gastric ulcer ($n = 12$), duodenal ulcer or duodenitis ($n = 67$) and non-cardia gastric carcinoma ($n = 20$). This classification was also confirmed by histological examinations. These patients have been referred for upper gastrointestinal endoscopy to the Gastroenterology Section of the University Hospitals (Namazi and Shahid Faghihi) of Shiraz University of Medical Sciences between 2002 and 2005. Patients who had received non-steroidal anti-inflammatory drugs were excluded. *H pylori* strains were successfully isolated from the gastric biopsies of 286 patients (150 males, 136 females; median age 45.3 ± 16.6 years). The present study was approved by the local ethics committee.

Bacterial culture and histological examination

Biopsy specimens were taken from the antrum and corpus of the stomach. These specimens were used for the rapid urease test, bacterial culture, and histological assessment. After 5 d of culture on selective agar plates, the organisms were identified as *H pylori* by Gram staining, colony morphology, and positive oxidase, catalase and urease reactions.

Preparation of patients and *H pylori* genomic DNA

After 3-5 d of culture, *H pylori* colonies were pooled from the plates and washed using phosphate-buffered saline. *H pylori* genomic DNA was prepared after bacterial cell lysis using SDS and proteinase K solution and phenol-chloroform extraction. Patients genomic DNA was extracted from EDTA anticoagulated peripheral blood leucocytes using a salting out method. The DNA samples were maintained at -70°C until use in polymerase chain reaction (PCR).

Analysis of IL-8 and bacterial *vacA* and *cagA* genotypes

All primer sets used were selected from the published literature and are shown in Table 1. An allele-specific oligonucleotide polymerase chain reaction (ASO-PCR) was used to detect the polymorphism at position -251 of the IL-8 gene^[30]. As an internal control, the β -globin specific primers were included in the ASO-PCR (Table 1). For IL-8 genotyping, 10 μL of PCR reaction mixture consisting of 250 ng of genomic DNA, 200 $\mu\text{mol/L}$ dNTPs, 2.25 mmol/L MgCl_2 , 1 \times Taq DNA polymerase buffer, 2 units of Taq DNA polymerase (Boehringer Mannheim, Germany), 10 pmol of each test primer and 5 pmol of internal control primers were employed. Then, a touch-down procedure was followed that consisted of 25 s at 95°C , annealing for 45 s at temperatures decreasing from 68°C (four cycles) to 61°C (20 cycles), and an extension step at 72°C for 40 s. The annealing temperature for the remaining 5 cycles was 58°C for 40 s. For *cagA* and *vacA* genes the PCR was performed using a thermal cycler (Master Cycler; Eppendorf, Germany) under the following conditions: an initial denaturation for 5 min at 94°C , 35 cycles of 60 s at 94°C , 60 s at 57°C and 60 s at

Table 1 Primer sequences used for detection of *cagA* gene status and IL-8 or *vacA* gene polymorphisms

Locus	Primers	Size (bp)
IL-8 -251	Common primer, 5'-tgc ccc ttc act ctg tta ac-3' A allele, 5'-cca caa ttt ggt gaa tta tca at-3' T allele, 5'-cca caa ttt ggt gaa tta tca aa-3'	336
β -globin	5'-aca caa ctg tgt tca cta gc-3' 5'-caa ctt cat cca cgt tca cc-3'	100
<i>cagA</i>	5'-aat aca cca acg cct cca ag-3' 5'-ttg ttg ccg ctt ttg ctc tc-3'	400
<i>vacA</i>		
S1 and s2	5'-ctg ctt gaa tgc gcc aaa c-3' 5'-atg gaa ata caa caa aca cac-3'	s1 = 259 s2 = 286
m1 and m2	5'-gcg tct aaa taa ttc caa gg-3' 5'-caa tct gtc caa tca agc gag-3'	m1 = 570 m2 = 645
<i>glmM</i>	5'-aag ctt tta ggg gtg tta ggg gtt t-3' 5'-aag ctt act ttc taa cac taa cgc-3'	294

72°C, with a final extension step at 72°C for 5 min. The PCR system for *cagA* contained 10 × PCR buffer, 2.5 μ L; MgCl₂, 1.2 mmol/L; dNTP, 200 μ mol/L; *cagA* specific primers, 10 pmol; Taq DNA polymerase, 1.0 U; and the DNA template, 50 ng. To confirm the identification of the bacteria as *H. pylori*, 10 pmol of *glmM* specific primers were included in each PCR reaction for *cagA* gene (*glmM* is a conserved gene formerly known as *ureC*). For detection of *vacA* polymorphisms a multiplex PCR was performed using the specific primers for amplification of s and m genes (Table 1). The PCR system for *vacA* genotyping was similar to *cagA* genotyping, PCR products were examined by 2% agarose gel electrophoresis and photographed using an ultraviolet reflection analyzer.

Statistical analysis

Fisher's exact test and the χ^2 test were used to analyze the data from different disease groups. All tests were performed two tailed with a confidence interval (CI) of 95%. A *P*-value of less than 0.05 was accepted as statistically significant. The Statistical Package for the Social Sciences (SPSS) version 11.5 was used for statistical analysis.

RESULTS

Prevalence of the *cagA* and *vacA* gene in different disease groups

Table 2 shows the distribution of the 286 strains according to their *cagA* and *vacA* types in different patient groups. To detect the *cagA* subtype of *H. pylori*, *cagA* specific PCR was performed on extracted bacterial DNA from patients with gastric diseases. Amplified *cagA* DNA fragments were detected in 219/286 (76.6%) of our *H. pylori*. Sixty seven patients (23.4%) were *cagA*-, although they were *glmM*+ or *vacA*+. When the patients with different clinical manifestations were compared according to the presence of the *cagA* gene (Table 2), insignificant differences in *cagA* status were found among patients with peptic ulcer, gastritis and gastric cancer ($\chi^2 = 1.9$; *P* = 0.38). Also, when the frequency of *cagA*+ and *cagA*- subtypes were compared among patients with gastritis and gastric ulcer

Table 2 IL-8 -251 A/T polymorphism, *cagA* status and *vacA* gene polymorphism in patients with gastric diseases

Locus	Genotype	Patients Gastritis <i>n</i> (%)	Peptic Ulcer <i>n</i> (%)	Gastric Cancer <i>n</i> (%)	<i>P</i> value
IL-8 -251	AA	22 (14.4)	14 (23.0)	9.0 (47.4)	0.013
	AT	74 (48.4)	28 (45.9)	6.0 (31.6)	
	TT	57 (37.3)	19 (31.1)	4.0 (21.1)	
<i>cagA</i>	+	148 (74.4)	54 (80.6)	17 (85.0)	0.39
	-	51 (25.6)	13 (19.4)	3.0 (15.0)	
<i>vacA</i>	s1m1	48 (26.5)	21 (32.3)	12.0 (66.7)	0.001
	s1m2	74 (40.9)	33 (50.8)	3.0 (16.7)	
	s2m2	59 (32.6)	11 (16.9)	3.0 (16.7)	

($\chi^2 = 1$; *P* = 0.3), gastritis and peptic ulcer ($\chi^2 = 0.75$; *P* = 0.38), gastritis and cancer ($\chi^2 = 0.61$; *P* = 0.42) or between peptic ulcer and gastric cancer ($\chi^2 = 0.01$; *P* = 0.75), the difference always remained insignificant. In the case of *vacA* gene, three of the four possible combinations of signal sequence and middle-region types were identified. The s1/m1 type was found in 81/264 (30.7%) of the isolates, the s1/m2 type in 110/264 (41.7%) of the isolates, and the s2/m2 type in 73/264 (27.7%) of the isolates. The distribution of *vacA* genotypes (s1/m1, s1/m2 or s2/m2) were significantly different among peptic ulcer, gastritis and gastric cancer patients ($\chi^2 = 17.8$; *P* = 0.001). Interestingly, the difference in distribution of three different *vacA* genotypes between patients with gastric cancer and gastritis ($\chi^2 = 12.57$; *P* = 0.0018) was more significant than the difference in distribution of *vacA* genotypes between patients with gastric cancer and peptic ulcer ($\chi^2 = 7.97$; *P* = 0.018). In fact, the frequency of s1m1 genotype was notably higher in gastric cancer patients (66.7%) compared to those with gastritis or peptic ulcer (26.5% and 32.3%, respectively). Furthermore, similar to previous reports^[15], there is a strong statistical linkage between the s1 genotype of *vacA* and the presence of the *cag* island ($\chi^2 = 27.95$; *P* = 0.0000001; OR = 4.99, 95% CI = 2.61-9.58). Similarly, the s2 genotype is associated with the lack of the *cag* island. In fact, strains that are *cag*+ are more likely to possess the *vacA* s1 allele than *cag*- strains.

Prevalence of the IL-8 -251 A/T polymorphism in different disease groups

The allelic frequencies of IL-8 -251 A/T polymorphism and genotype distributions are given in table 2. The IL-8 -251 A/T polymorphism showed no evidence of deviation from the Hardy-Weinberg equilibrium, with a non-significant χ^2 value ($\chi^2 = 0.05$, *P* = 0.4). Interestingly, there was a significant difference in the frequency of IL-8 -251 A/T genotypes between patients with gastric cancer and those with benign diseases ($\chi^2 = 10.47$; *P* = 0.005). Moreover, when the patients were categorized to high producer (AA) and intermediate + low producer (AT + TT) genotypes, a more meaningful difference in the frequency of IL-8 -251 A/T genotypes between patients with gastric cancer and benign diseases was noticeable (OR = 4.45, 95% CI = 1.53-12.94; $\chi^2 = 8.58$, *P* = 0.003). In fact, the prevalence of AA genotype in gastric cancer

patients was 47.4% compared to 16.8% in benign diseases. While comparing different patient groups, no significant differences were demonstrated in frequencies of IL-8 -251 A/T genotypes between patients with peptic ulcer and gastritis ($\chi^2 = 2.4$; $P = 0.25$) or gastric cancer ($\chi^2 = 4.22$; $P = 0.12$). However, IL-8 -251 A/T polymorphism showed a significant difference between patients with gastric cancer and gastritis ($\chi^2 = 12.5$; $P = 0.001$).

DISCUSSION

After exposure to *H pylori*, the clinical manifestations are variable and depend on host and pathogen factors. There is no information on the prevalence and role of *H pylori* genotypes and/or the role of IL-8 -251 A/T genotypes in the disease outcome of Iranian patients. Therefore, in the present study, we determined the presence of the *cagA* gene (as a marker of *cag* pathogenicity island) and the genotypes of *vacA* gene in the infecting strains along with the distribution of host IL-8 genotypes in relation to the occurrence of different clinical manifestations in Iranian patients with *H pylori* infection. It has been shown that exposure of gastric epithelial cells to *cag+* *H pylori* strains can activate the proto-oncogenes *c-fos* and *c-jun*, a crucial step in the development of *H pylori*-related neoplasia^[31]. The presence of *cagA* has been statistically associated with duodenal ulceration, gastric mucosal atrophy, and gastric cancer^[7-9], although some studies deny this association^[32-34]. Audibert and her colleagues reported that *cagA* status is not sufficient to predict the IL-8 induction ability of *H pylori* and is not correlated with the presence or absence of ulcer^[35]. In a series of patients from Taiwan, the presence of *cagA* gene in the PAI also showed no relationship to the type of disease and/or the histological features of the patients^[36]. The results of the present study also shows that the prevalence of *cagA* is not significantly different among different disease groups ($P > 0.05$), which is in accordance with the results of other Asian countries. Therefore, the *cag*-PAI may not be the principal virulence factor, as suggested by the absence or sporadic distribution of the *cag*-PAI genes among strains from varied clinical outcomes. However, considering the high prevalence of *cag+* strains in Iranian patients (76.6%), the relationship of *cag* status with disease type is more difficult to establish in our population. Therefore, larger sample sizes are recommended for such studies.

Furthermore, the lack of association may be due to the fact that the development of cancer or ulcer disease is a complex process that also involves factors other than the *cag*-PAI, such as *vacA*. Certain *vacA* genotypes causing a high vacuolating activity are correlated with increased disease severity in humans^[15]. Several studies have also shown that the presence of *vacA* is associated with peptic ulcer diseases^[14,17-18]. The *vacA* gene displays a considerable polymorphism, especially in the signal region (genotypes s1 and s2) and in a mid region (genotypes m1 and m2). Vacuolating activity is higher in s1/m1 genotypes than in s1/m2 genotypes, and is absent in s2/m2 genotypes^[15]. Consequently, *vacA* s1/m1 strains cause more direct epithelial damage and are more frequently associated with

peptic ulceration and gastric carcinoma^[9,17,18]. The results of the present study also show a significant difference in *vacA* genotype distribution between gastric cancer and gastritis ($P = 0.0018$) or peptic ulcer ($P = 0.018$) patients. The marked increase of s1m1 genotype in gastric cancer patients (66.7%) compared to those in patients with gastritis (26.5%) or peptic ulcer (32.3%) confirms the pathogenic role of this virulence determinant in Iranian patients. However, different disease outcomes were encountered in subjects infected with *H pylori* strains sharing the same virulent *vacA* genotype, s1m1. The different outcomes of *H pylori* infection may depend not only on other bacterial factors but also on the different genetic background of the host. Concerning host genetic factors, Thye *et al* performed a genome wide screen analysis to identify the genetic factor(s), which define susceptibility to *H pylori* infection^[37] and suggested the presence of a possible linkage with chromosomes^[4]. Considering the location of the human IL-8 gene on chromosome 4 (4q13-q21), the results of their study may support the hypothesis that the IL-8 gene polymorphism is probably associated with *H pylori* induced gastrointestinal diseases. Interestingly, the inheritance of the IL-8 -251A allele was associated with progression of gastric atrophy in patients with *H pylori* infection and increased the risk of gastric cancer in Japanese and Chinese people^[28,29,38]. Our results also indicate that gastric cancer is significantly associated with the functional polymorphism in the promoter region of the IL-8 gene. Specifically, individuals genetically predisposed to produce more IL-8 are at a higher risk of developing gastric cancer. The finding that there was an increased risk of gastric cancer in high IL-8 producers was in agreement with the concept that IL-8 may influence the risk of developing gastric cancer by altering the quality and vigor of inflammatory responses produced by the host after exposure to *H pylori*. In addition, IL-8 stimulated neutrophils to synthesize active radicals such as nitric oxide^[25]. These radicals by their mutagenic potential^[39] could cause mutations in gastric epithelial cells. In addition, IL-8 by inducing angiogenesis would be one of the important factors in gastric carcinogenesis. In support of this hypothesis, the expression of IL-8 has been associated with increased vascularization and poor prognosis in gastric carcinoma^[40,41]. Thus, inheritance of the high producer allele of IL-8 (carriers of -251 A allele) may induce chronic gastritis, which may then be followed by the development of gastric cancer.

In conclusion, similar to studies performed in China and Japan, the association between *cagA* positivity and virulence of *H pylori* strains was equally frequent among Iranian patients with different disease types. Moreover, the present study provides further evidence that in addition to genetic polymorphism of the *vacA* gene in the pathogen, genetically determined differences in IL-8 production via promoter polymorphisms could contribute to individual susceptibility to gastric cancer development after *H pylori* infection in Iranian patients.

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Preliminary report of hepatitis B virus genotype prevalence in Iran

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INTRODUCTION

Human hepatitis B virus (HBV), which is the prototype member of the family Hepadnaviridae, is a circular, partially double-stranded DNA virus of approximately 3200 nt^[1]. This highly compact genome contains the four major open reading frames encoding the envelope (preS1, preS2 and surface antigen HBsAg), polymerase (HBPol) and X (HBX) proteins, respectively^[2]. HBV is an etiologic agent of acute and chronic liver disease, including fatal fulminant hepatitis, liver cirrhosis and hepatocellular carcinoma^[3-6]. Over 2 billion people worldwide have been exposed to HBV and 350 million are chronic carriers of HBV^[7-9].

In 1988, HBV was classified into four genotypes by a sequence divergence in the entire genome exceeding 8%, and designated by capital letters of the alphabet from A to D^[10,11]. In 1994, Norder *et al*^[12] found an additional two HBV genotypes by the same criteria, and named them E and F, respectively. Genotype G was reported recently in 2000^[2] and genotype H, which is phylogenetically closely related to genotype F, was proposed in 2002^[11]. HBV genotypes have distinct geographical distribution^[2,7,13,14].

In general, genotype A is pandemic, but most prevalent in North West Europe, North America, Central Africa^[2,13] and India^[7]. Genotypes B and C are prevalent in Asia^[7,8,13,15], especially in populations of Eastern Asia and the far East origin^[3,16]. Genotype D is also more or less pandemic, but is predominant in the Mediterranean area and the Middle East^[2,3,16]. Genotype E is restricted to Africa and genotype F is found in Central and South America^[7,8,13,16]. Genotype G has been recently identified in France and North America^[7,8]. It has been reported that there are remarkable differences in the clinical and virologic characteristics between the patients with different genotypes^[17,18,19]. According to Iranian studies^[20], over 35% of Iranians have been exposed to HBV, approximately 2% are chronic carriers. Compared to the United States where HBV infection is responsible for 25% of chronic hepatitis, HBV accounts for up to 70%-80% of chronic hepatitis cases in Iran, indicating that HBV alone is the leading

Abstract

AIM: To determine the prevalence of hepatitis B virus (HBV) genotypes in Iranian hepatitis B surface antigen (HBsAg) carriers, chronic hepatitis B and cirrhotic patients.

METHODS: A total of 109 HBsAg-positive patients were included in this study. HBV genotypes were determined by using INNO-LiPA methodology which is based on the reverse hybridization principle.

RESULTS: The distribution of patients with different stages of liver disease was as follows: 95 (86.4%) chronic hepatitis, 11 (10%) liver cirrhosis, and 3 (2.7%) inactive carrier. Of the chronic hepatitis and liver cirrhosis patients, 26.4% were HBeAg-positive while 70% were HBeAg-negative. Genotype D was the only detected type found in all patients.

CONCLUSION: Classifying HBV into genotypes has to be cost-effective and clinically relevant. Our study indicates that HBV genotype D prevails in the Mediterranean area, Near and Middle East, and South Asia. Continued efforts for understanding HBV genotype through international co-operation will reveal further virological differences of the genotypes and their clinical relevance.

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Key words: Hepatitis B virus; Genotype; Chronic Hepatitis B; Cirrhosis

cause of chronic liver disease in Iran^[21]. Until now, to our knowledge, no data regarding HBV genotype is available, and also the genotypes distribution of HBV and genotype-related differences with the liver disease are still unclear in Iran. In this study, we therefore determined the prevalence of HBV genotypes in Iranian hepatitis B surface antigen (HBsAg) carriers, chronic hepatitis B and cirrhotic patients.

MATERIALS AND METHODS

This cross-sectional study was performed in Tehran Hepatitis Center in 2004. A total of 109 patients with hepatitis B surface antigen (HBsAg) positive for at least 6 mo were enrolled in this study. Of the 109 patients, 95 were classified as chronic hepatitis, defined as HBsAg positive with or without the presence of HBeAg, high level of HBV-DNA (> 100 000 copies/mL) detected by Amplicor HBV monitor, persistent or intermittent elevation in ALT levels and compatible liver biopsy. Three were inactive carriers characterized by persistent HBV infection of liver without significant, ongoing necro-inflammatory disease. Eleven had liver cirrhosis characterized by clinical evidence (splenomegaly, ascitis) and paraclinical results, including low platelet count, prolongation of prothrombin time and esophageal varices on upper gastrointestinal endoscopy. Liver cirrhosis was confirmed by liver biopsy. Patients were excluded if they were co-infected with hepatitis C virus (HCV), hepatitis D virus (HDV) or human immunodeficiency virus (HIV).

The following parameters were recorded for each patient from patient's document in Tehran Hepatitis Center: sex, age, stage of liver disease, alanine aminotransferase (ALT) level, aspartate transaminase (AST) and presence of hepatitis B virus E antigen and anti-hepatitis B virus E antibodies.

HBV genotypes were determined by using INNO-LiPA methodology (LiPA, INNO-LiPA HBV genotyping assay, Innogenetics N.V., Ghent, Belgium). The INNO-LiPA HBV genotyping assay is a line probe assay designated to identify hepatitis B virus genotypes A to G by detection of type-specific sequences in the HBV-pol gene domain B to C. This method is based on the reverse hybridization principle. Biotinylated DNA material generated from the HBsAg open reading frame was hybridized with specific oligonucleotide probes immobilized as parallel lines on membrane-based strips. After hybridization, unhybridized DNA was washed from the strip, alkaline phosphatase-labeled streptavidin was added and bounded to any biotinylated hybrid previously formed. Incubation with BCIP/NBT chromogen resulted in a purple/brown precipitate. Amplification of appropriate the HBV genomic region was performed using the INNO-LiPA HBV DR amplification kit. The INNO-LiPA HBV genotyping strip contains 1 red marker line, 2 control lines, and 14 parallel probe lines. The conjugate control line is a control for the color development reaction and the amplification control line contains universal HBV probes to check for the presence of amplified the HBV genomic material.

Statistical analysis

Data were analyzed with SPSS 11.5 software (SPSS Inc. Chicago, Illions, USA) using Student's *t* test, χ^2 test and Fisher's exact test.

RESULTS

A total of 109 patients with a mean age of 37.17 ± 11.75 years, including 13% females and 87% males, were enrolled in this HBV genotype study. The distribution of patients in different stages of liver disease was as follows: 95 (86.4%) chronic hepatitis, 11 (10%) liver cirrhosis, and 3 (2.7%) inactive carrier. Of the chronic hepatitis and liver cirrhosis patients, 26.4% were HBeAg-positive and 70% were HBeAg-negative. The mean serum ALT, AST, and ALP levels were 126.08 IU/L (88.46-163.71), 86.46 IU/L (49.54-123.39), 173.34 IU/L (152.74-193.94), respectively.

Genotype D was the only detected type found in all patients. Mean age of patients was significantly higher in the anti-HBe-positive group as compared with the HBe-Ag-positive group ($P = 0.000$). Also, the number of the patients in the anti-HBe-positive group was significantly higher than the HBe-Ag-positive group ($P = 0.019$; Fisher's exact test). Moreover, significant difference was found between the mean age of patients with different stages of liver disease. None of the patients in the HBe-Ag-positive group had a normal ALT level. Most of the patients in the chronic hepatitis stage had an abnormal ALT level in comparison with the liver cirrhosis stage ($P = 0.024$; Fisher's exact test).

DISCUSSION

Classifying HBV into genotypes has to be cost-effective and clinically relevant. It is imperative to collect more information on HBV genotypes from all over the world to reach a decision on their clinical utility^[11].

Data on the relation among the HBV genotypes, their pathogenicity in chronic liver disease including hepatocellular carcinoma and their effect on therapy are awaited with great interest, especially in Asia which is an endemic region of blood-borne hepatitis viruses^[15].

Presently, based on an intergroup divergence of 8% or more in the complete nucleotide sequence, HBV can be classified into eight genotypes A-H, and different HBV genotypes are dominant in various parts of the world^[14,16]. The most important finding of our study was that the only HBV genotype D was detected in all patients. The pattern of distribution of genotypes seemed to be simpler and was predominantly centralized into genotype D in all forms of the chronic HBV infection. The results of this study concur with previous studies, indicating that HBV genotype D prevails in the Mediterranean area, Near and Middle East, and South Asia^[3,4,16]. For example, the result of a similar study performed in Turkey showed all 44 patients studied had genotype D^[16]. Another study in Yemen demonstrated that genotype D was the dominant genotype in a settled population, while genotype A was

found only in communities with continuing African links^[22]. In addition, one study in Egypt revealed genotype D was the most prevalent HBV genotype^[23]. On the contrary, genotypes A, B and C were found to be predominant in Pakistan^[24]. According to recent studies, genotype D in Asia is associated with more severe disease and may predict occurrence of hepatocellular carcinoma in younger patients^[9].

After all, only less than 1 000 of the 350 million persistent HBV infections have yet been genotyped. Continued efforts for understanding HBV genotypes through international co-operation will reveal further virological differences of the genotypes and their clinical relevance.

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RAPID COMMUNICATION

Effect of Fructus Psoraleae on motility of gallbladder isolated smooth muscle strips from guinea pigs

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Abstract

AIM: To observe the effect of Fructus Psoraleae on motility of isolated gallbladder muscle strips of guinea pigs and its mechanism.

METHODS: Guinea pigs were hit to lose consciousness and the whole gallbladder was removed quickly. Two or three smooth muscle strips (8 mm × 3 mm) were cut along a longitudinal direction. The mucosa was gently removed. Every longitudinal muscle strip was suspended in a tissue chamber which was continuously perfused with 5 mL Krebs solution (37°C), pH 7.4, and aerated with 950 mL/L O₂ and 50 mL /L CO₂. The isometric response was recorded with an ink-writing recorder. After 2 h equilibration under 1 g-load, 50 μL Fructus Psoraleae (10, 20, 70, 200, 700, 1000 g/L) was added cumulatively into the tissue chamber in turn every 2 min to observe their effects on gallbladder muscle strips (cumulating final concentration of Fructus Psoraleae was 0.1, 0.3, 1.0, 3.0, 10.0, 20.0 g/L). The antagonists, including 4-DAMP, benzhydramine, hexamethonium, phentolamine, verapamil and idomethine were given 2 min before Fructus Psoraleae respectively to investigate the mechanisms involved.

RESULTS: Fructus Psoraleae dose-dependently increased the resting tension ($r = 0.992, P < 0.001$), decreased the mean contractile amplitude ($r = 0.970, P < 0.001$) and meanwhile increased the contractile frequency of the gallbladder muscle strip *in vitro* ($r = 0.965, P < 0.001$). The exciting action of Fructus Psoraleae on the resting tension could be partially blocked by 4-DAMP (the resting tension decreased from 1.37 ± 0.41 to 0.70

$\pm 0.35, P < 0.001$), benzhydramine (from 1.37 ± 0.41 to $0.45 \pm 0.38, P < 0.001$), hexamethonium (from 1.37 ± 0.41 to $0.94 \pm 0.23, P < 0.05$), phentolamine (from 1.37 ± 0.41 to $0.89 \pm 0.22, P < 0.01$) and verapamil (from 1.37 ± 0.41 to $0.94 \pm 0.26, P < 0.05$). But the above antagonists had no significant effect on the action of Fructus Psoraleae-induced mean contractile amplitude ($P > 0.05$). Moreover, the increase of the contractile frequency due to Fructus Psoraleae was inhibited by 4-DAMP (decreased from 8.3 ± 1.2 to $6.8 \pm 0.5, P < 0.01$) and hexamethonium (from 8.3 ± 1.2 to $7.0 \pm 0.9, P < 0.05$). Idomethine had no significant effect on the Fructus Psoraleae-induced responses ($P > 0.05$).

CONCLUSION: Fructus Psoraleae enhances the motility of isolated gallbladder muscle strips from guinea pigs, in a dose-dependent manner. The effect of Fructus Psoraleae is partly related to M₃, N receptor, α receptor, H₁ receptor, Ca²⁺ channel, but not related to prostaglandin.

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Key words: Fructus Psoraleae; Gallbladder smooth muscle strips; M₃, N, α, H₁ receptors; Ca²⁺ channel; Prostaglandin

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INTRODUCTION

Fructus Psoraleae is the dry fruit of the leguminous plant *Psoralea corylifolia* L. In recent years, researchers from home and abroad have made extensive studies on its chemical composition and extraction^[1-4]. Studies on the pharmacological action of Fructus Psoraleae have been focused on its therapeutic effect on leucopenia, uterine bleeding, chronic bronchitis and psoriasis^[5-8]. However, reports of the effect and mechanism of Fructus Psoraleae on the gallbladder smooth muscle strips *in vitro* are rare. In this experiment, we observed the effects of Fructus Psoraleae on the gallbladder muscle strips from guinea pigs and studied the possible mechanisms involved.

Table 1 Effect of Fructus Psoraleae on resting tension (g) of isolated gallbladder muscle strip after pretreated with antagonists (means \pm SD)

Resting tension (g)	Fructus Psoraleae (g/L)						
	0	0.1	0.3	1	3	10	20
Pso	0.00 \pm 0.00	0.00 \pm 0.00	0.02 \pm 0.04	0.09 \pm 0.04	0.24 \pm 0.08 ^a	0.88 \pm 0.35 ^d	1.37 \pm 0.41 ^d
Ben + Pso	0.00 \pm 0.00	0.00 \pm 0.00	0.02 \pm 0.04	0.07 \pm 0.06	0.14 \pm 0.08 ^c	0.29 \pm 0.17 ^{b,h}	0.45 \pm 0.38 ^{d,h}
Phe + Pso	0.00 \pm 0.00	0.00 \pm 0.00	0.01 \pm 0.04	0.07 \pm 0.07	0.17 \pm 0.08 ^b	0.46 \pm 0.11 ^{d,h}	0.89 \pm 0.22 ^{d,f}
4-DAMP + Pso	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00	0.01 \pm 0.04 ^f	0.09 \pm 0.07 ^f	0.30 \pm 0.18 ^{d,h}	0.70 \pm 0.35 ^{d,h}
Hex + Pso	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00	0.05 \pm 0.08	0.16 \pm 0.14 ^a	0.42 \pm 0.20 ^{c,h}	0.94 \pm 0.23 ^{c,d}
Ido + Pso	0.00 \pm 0.00	0.00 \pm 0.00	0.01 \pm 0.02	0.09 \pm 0.06	0.21 \pm 0.12 ^a	0.71 \pm 0.23 ^d	1.31 \pm 0.41 ^d
Iso + Pso	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00	0.01 \pm 0.03 ^f	0.10 \pm 0.06 ^f	0.38 \pm 0.19 ^{c,h}	0.94 \pm 0.26 ^{c,d}

^a $P < 0.05$, ^b $P < 0.01$, ^d $P < 0.001$ vs control (under 1-g initial load the gallbladder spontaneous contraction when Fructus Psoraleae was 0 g/L). The resting tension of each strip in control was 0 ($n = 8$). ^c $P < 0.05$, ^f $P < 0.01$, ^h $P < 0.001$ vs Fructus Psoraleae (the resting tension of adding each concentration of Fructus Psoraleae) ($n = 8$). Pso: Fructus Psoraleae; Ben: Benzhydramine; Phe: Phentolamine; Hex: Hexamethonium; Ido: Idomethine; Iso: Verapamil.

MATERIALS AND METHODS

Materials

Fructus Psoraleae was ground into coarse powder, boiled, filtered and made into (1000 g/L) extract (the drug was prepared and tested by Gansu Institute for Drug Control), and then diluted to 10, 20, 70, 200, 700, 1000 g/L solutions respectively. The antagonists are as follows: 4-DAMP (1 μ mol/L) (Sigma Chemicals Company), hexamethonium (10 μ mol/L) (Sigma Chemicals Company), phentolamine (1 μ mol/L) (Beijing No 13 Pharmaceutical Factory), verapamil (0.05 μ mol/L) (Lanzhou Pharmaceutical Factory), idomethine (1 μ mol/L) (Beijing Two-bridge Pharmaceutical Factory), and benzhydramine (1 μ mol/L) (Jiangsu Taicang Pharmaceutical Factory). Guinea pigs of either sex, weighing between 350 and 450 g [purchased from Animal Center of Lanzhou Veterinary Institute, laboratory animal certificate FCXK (Gan2004-0005)]. The following equipments were used: JZ-BK external isometric force transducer (BK Company), LMS-ZB two channels recorder (Chengdu Equipment Factory, China).

Methods

Guinea pigs were fasted with free access to water for 24 h. They were hit on the head to become unconscious. The whole gallbladder was removed, quickly transferred to Krebs solution and rinsed. The wall of the gallbladder was incised from the end of the cystic duct to the base to make two or three longitudinal smooth muscle strips (8 mm \times 3 mm). The mucosa was gently removed with forceps. Every longitudinal muscle strip was suspended in a tissue chamber which was continuously perfused with 5 mL Krebs solution (37°C), pH 7.4, and aerated with 950 mL/L O₂ and 50 mL /L CO₂. One end of the strip was fixed to a hook on the bottom of the chamber. The other end was connected to an external isometric force transducer (JZ-BK, BK). The strip was subjected to 1 g load tension and washed with 5 mL Krebs solution every 20 min. Motility of gallbladder strips in tissue chambers was simultaneously recorded by electrophysiograph (LMS_ZB, Chengdu), including the resting tension, the mean contractile amplitude, and the contractile frequency^[9]. After 2 h equilibration, 10, 20, 70, 200, 700, 1000 g/L Fructus Psoraleae was added

cumulatively in turn every 2 min to observe their effects on contractility of gallbladder. The 4-DAMP (1 μ mol/L), benzhydramine (1 μ mol/L), hexamethonium (10 mol/L), phentolamine (1 μ mol/L), verapamil (0.05 μ mol/L) and idomethine (1 μ mol/L) were added 2 min before Fructus Psoraleae was added to investigate whether the actions of Fructus Psoraleae were mediated *via* M₃, N receptor, α receptor, H₁ receptor, Ca²⁺ channel and prostaglandin(PG).

Statistical analysis

The data were presented as mean \pm SD and analyzed with one-way ANOVA and correlation. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of Fructus Psoraleae and antagonists plus Fructus Psoraleae on the resting tension of gallbladder muscle strips

Fructus Psoraleae dose-dependently increased the resting tension of gallbladder muscle strips *in vitro* ($r = 0.992$, $P < 0.001$). The 4-DAMP (1 μ mol/L), benzhydramine (1 μ mol/L), hexamethonium (10 μ mol/L), phentolamine (1 μ mol/L), verapamil (0.05 μ mol/L) and idomethine (1 μ mol/L) had no significant effects on the resting tension of gallbladder muscle strips. But when given 2 min before the administration of Fructus Psoraleae (0.1, 0.3, 1.0, 3.0, 10.0, 20.0 g/L), 4-DAMP, benzhydramine, hexamethonium, phentolamine and verapamil partly blocked the enhancing action of Fructus Psoraleae on the resting tension of gallbladder muscle strips. However, idomethine had no significant action on the increasing effect of Fructus Psoraleae on resting tension (Table 1).

Effect of Fructus Psoraleae and antagonists plus Fructus Psoraleae on the mean contractile amplitude of gallbladder muscle strips

Fructus Psoraleae dose-dependently decreased the mean contractile amplitude of gallbladder isolated smooth muscle strips ($r = 0.970$, $P < 0.001$). The 4-DAMP (1 μ mol/L), benzhydramine (1 μ mol/L), hexamethonium (10 μ mol/L), phentolamine (1 μ mol/L), verapamil (0.05 μ mol/L) and idomethine (1 μ mol/L) had no significant effects on the mean contractile amplitude of gallbladder muscle strips. When

Table 2 Effect of Fructus Psoraleae on the contractile amplitude (mm) of isolated gallbladder muscle strip after pretreated with antagonists (means \pm SD)

Amplitude (mm)	Fructus psoraleae (g/L)						
	0	0.1	0.3	1	3	10	20
Pso	4.54 \pm 0.64	4.23 \pm 0.78	4.24 \pm 0.84	4.07 \pm 0.72	3.71 \pm 0.59 ^a	2.36 \pm 0.77 ^d	1.72 \pm 0.74 ^d
Ben + Pso	4.70 \pm 0.57	4.43 \pm 0.80	4.19 \pm 0.88	4.09 \pm 0.97	3.93 \pm 0.97	3.55 \pm 0.93 ^a	2.37 \pm 0.83 ^d
Phe + Pso	4.71 \pm 1.37	4.47 \pm 1.42	4.64 \pm 1.32	4.49 \pm 1.35	4.17 \pm 1.41	3.36 \pm 1.43 ^a	2.15 \pm 0.65 ^d
4-DAMP + Pso	4.91 \pm 0.60	4.58 \pm 0.71	4.23 \pm 0.60	4.27 \pm 0.84	3.84 \pm 0.81 ^a	3.70 \pm 1.28 ^b	2.34 \pm 1.07 ^d
Hex + Pso	5.48 \pm 1.57	5.13 \pm 1.64	5.34 \pm 1.60	4.86 \pm 1.63	4.80 \pm 1.46	3.43 \pm 1.10 ^b	1.93 \pm 1.02 ^d
Ido + Pso	5.40 \pm 1.30	5.01 \pm 1.37	4.83 \pm 1.33	4.62 \pm 1.42	4.16 \pm 1.29	3.39 \pm 1.48 ^b	2.24 \pm 1.25 ^d
Iso + Pso	4.47 \pm 1.23	4.42 \pm 1.37	4.60 \pm 1.56	4.29 \pm 1.32	4.00 \pm 1.08	3.98 \pm 1.74	2.80 \pm 1.57 ^b

^a $P < 0.05$, ^b $P < 0.01$, ^d $P < 0.001$ vs control (under 1-g initial load the gallbladder spontaneous mean contraction amplitude when Fructus Psoraleae was 0 g/L) ($n = 8$).

Table 3 Effect of Fructus Psoraleae on the contractile frequency(waves/min) of isolated gallbladder muscle strip after pretreated with antagonists (means \pm SD)

Frequency (w/min)	Fructus psoraleae (g/L)						
	0	0.1	0.3	1	3	10	20
Pso	3.3 \pm 0.5	3.3 \pm 0.7	3.3 \pm 0.6	3.8 \pm 0.9	4.6 \pm 0.7 ^b	7.3 \pm 1.2 ^d	8.3 \pm 1.2 ^d
Ben + Pso	3.4 \pm 0.5	3.6 \pm 0.4	3.6 \pm 0.4	3.7 \pm 0.4	4.6 \pm 0.4 ^d	6.3 \pm 0.5 ^d	7.3 \pm 0.8 ^d
Phe + Pso	3.5 \pm 0.7	3.9 \pm 0.8	3.9 \pm 0.8	4.4 \pm 1.0	5.1 \pm 0.8 ^b	7.1 \pm 1.2 ^d	9.1 \pm 1.1 ^d
4-DAMP + Pso	3.5 \pm 0.4	3.5 \pm 0.4	3.6 \pm 0.6	3.9 \pm 0.7	4.2 \pm 0.5 ^a	5.1 \pm 0.9 ^{d,h}	6.8 \pm 0.5 ^{d,f}
Hex + Pso	3.3 \pm 0.5	3.5 \pm 0.8	3.5 \pm 0.5	3.9 \pm 0.8	4.8 \pm 1.0 ^d	6.0 \pm 0.7 ^{c,d}	7.0 \pm 0.9 ^{c,d}
Ido + Pso	3.4 \pm 0.5	3.4 \pm 0.6	3.6 \pm 0.7	3.8 \pm 0.7	4.4 \pm 0.7 ^a	6.9 \pm 1.4 ^d	8.4 \pm 1.1 ^d
Iso + Pso	3.4 \pm 0.5	3.4 \pm 0.8	3.5 \pm 0.8	3.8 \pm 0.9	5.2 \pm 1.4 ^b	6.3 \pm 1.2 ^d	8.8 \pm 1.6 ^d

^a $P < 0.05$, ^b $P < 0.01$, ^d $P < 0.001$ vs control (under 1-g initial load the gallbladder spontaneous contraction frequency when Fructus Psoraleae was 0 g/L) ($n = 8$). ^c $P < 0.05$, ^f $P < 0.01$, ^h $P < 0.001$ vs Fructus Psoraleae (contraction frequency of adding each concentration of Fructus Psoraleae) ($n = 8$).

added 2 min before administration of Fructus Psoraleae (0.1, 0.3, 1.0, 3.0, 10.0, 20.0 g/L), none of the above antagonists showed significant action on the decreasing effect of Fructus Psoraleae on the mean contractile amplitude (Table 2).

Effect of Fructus Psoraleae and antagonists plus Fructus Psoraleae on the contractile frequency of gallbladder muscle strips

Fructus Psoraleae dose-dependently increased the contractile frequency of gallbladder muscle strips from guinea pigs *in vitro* ($r = 0.965$, $P < 0.001$). The 4-DAMP (1 $\mu\text{mol/L}$), benzhydramine (1 $\mu\text{mol/L}$), hexamethonium (10 $\mu\text{mol/L}$), phentolamine (1 $\mu\text{mol/L}$), verapamil (0.05 $\mu\text{mol/L}$) and idomethine (1 $\mu\text{mol/L}$) had no significant effects on the contractile frequency of gallbladder muscle strips. When given 2 min before the administration of Fructus Psoraleae (0.1, 0.3, 1.0, 3.0, 10.0, 20.0 g/L), 4-DAMP and hexamethonium partly inhibited the action of Fructus Psoraleae on contractile frequency of gallbladder muscle strips; nevertheless, the other antagonists had no significant effects on the action of Fructus Psoraleae on the contractile frequency (Table 3).

DISCUSSION

Cholelithiasis is a common disease worldwide and many epidemiological studies have shown that the incidence of

cholelithiasis has been on a rapid increase in some regions of the world since the last decade. Motor dysfunction of the gallbladder plays an important role in cholelithiasis and cholecystitis^[10-12]. According to the literature, traditional Chinese medicine, western medicine and surgical management have been employed to treat cholelithiasis at home and abroad^[13-16]. Herbal medicine is characterized by having few side effects and good curative effects, which is gradually accepted by people over the world. Zhou^[17] reported that Fructus Psoraleae extract excited the intestinal canal *in vivo* and *in vitro*, and relaxed the uterus of guinea pigs. In the present study, we observed that Fructus Psoraleae significantly increased the resting tension and contractile frequency; meanwhile decreased the mean contractile amplitude of isolated gallbladder muscle strips of guinea pigs.

All smooth muscles in the gallbladder are involuntary and the nerves are controlled by both extrinsic and intrinsic nervous systems. Von Schrenck *et al*^[18] reported that gallbladder smooth muscle cells possess muscarinic receptors of the M₃ type, which mediate contraction. Chen *et al*^[19] reported the M₃ receptors are preferentially associated with the activation of phospholipase C, intracellular Ca²⁺ release and the calmodulin-dependent pathway. It has been identified that cholinergic N-receptor exists on the membrane of nerve ganglion cells of gallbladder smooth muscle. Our results showed that M₃

antagonist (4-DAMP) and hexamethonium (nicotinic cholinergic antagonist) partly blocked the enhancing action of Fructus Psoraleae on the resting tension and contractile frequency of gallbladder muscle strips, but not that of the contractile amplitude of the strips. These results suggested that Fructus Psoraleae excited gallbladder muscle strip *via* M₃ and ganglion N receptors.

Moreover, there were some relevant reports about histamine and histamine receptors. Jennings *et al.*^[20] proposed that histamine is distributed in the guinea-pig gallbladder and it could regulate contractile activity *via* activation of H₁ and H₂ but not H₃ receptor. Gallbladder muscle possesses stimulatory H₁ receptors and inhibitory H₂ receptors^[21]. The depolarization and associated contraction of gallbladder smooth muscle represent the net effect of activation of both H (1) (excitatory) and H (2) (inhibitory) receptors, with the H (2) receptor-mediated response involving the activation of K (ATP) channels^[22]. In this experiment, we observed that H₁ antagonist-benzhydramine partly inhibited the enhancing action of Fructus Psoraleae on the resting tension, but had no effect on the mean contractile amplitude and contractile frequency of gallbladder muscle strip. The results showed the excitatory action of Fructus Psoraleae on gallbladder muscle strip was possibly mediated *via* H₁ receptor. Yanaura *et al.*^[23] reported that contractions and relaxations produced by sympathomimetic amines are mediated by alpha-excitatory and beta-inhibitory adrenoceptors in the biliary system (gallbladder, common bile duct and sphincter of Oddi) of guinea-pigs. We also observed that adrenergic antagonist-phentolamine partly inhibited the enhancing action of Fructus Psoraleae on the resting tension, but had no effect on the mean contractile amplitude and contractile frequency of gallbladder muscle strip, and the results revealed that the excitatory action of Fructus Psoraleae on gallbladder muscle strip was possibly mediated *via* α receptor. Ca²⁺, which participates in excitation contraction coupling plays an important role in the contraction process of smooth muscle. The action potential in gallbladder smooth muscle (GBSM) is caused by Ca²⁺ entry through voltage-dependent Ca²⁺ channels (VDCC), which contributes to the GBSM contraction^[24]. Shimada^[25] believed the L-type Ca²⁺ current is dominant in gallbladder smooth muscle cells and may contribute to excitation-contraction coupling. In addition, our data showed that verapamil partly inhibited the exciting action of Fructus Psoraleae on the resting tension, suggesting that Fructus Psoraleae-induced gallbladder contraction was related to the Ca²⁺ channel. Verapamil is an L-type calcium channel blocker which inhibited the exciting effect induced by Fructus Psoraleae; whereas idomethine (prostaglandin enzyme suppressor) had no significant effects on the action of Fructus Psoraleae, indicating that the exciting action of Fructus Psoraleae on gallbladder smooth muscle strips was not related to prostaglandin (PG).

In summary, results from this study provide us new insights into the mechanisms underlying gallbladder motility and will be useful for further understanding of

biliary dyskinesia diseases and the treatment.

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K-19 mRNA RT-PCR in detecting micrometastasis in regional lymph nodes of gastric cancer

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Abstract

AIM: To investigate the value and prospect of RT-PCR in detecting micrometastasis in regional lymph nodes of gastric cancer.

METHODS: Histopathology was used and K19 mRNA expression was detected by RT-PCR in tumor tissues and lymph nodes from gastric cancer patients undergoing radical resection of gastric carcinoma.

RESULTS: K19 mRNA was expressed in all tumor specimens of 30 cases; of the 126 lymph nodes, 26 were histopathologically positive (20.6%), and 42 positive (33.3%) by RT-PCR. Amplification fragments of 460 and 540 bp were shown in all the tumor tissues and metastatic lymph nodes after K19 and β -actin RT-PCR, while only a 540 bp fragment appeared in the lymph nodes of non-tumor patients.

CONCLUSION: K19 mRNA RT-PCR is sensitive and specific in testing micrometastasis in regional lymph nodes of gastric cancer, and it is superior to routine histopathology.

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Key words: K-19 mRNA; RT-PCR; Micrometastasis; Gastric cancer

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INTRODUCTION

Gastrointestinal cancer is the most common malignant tumor of the digestive tract. For histologically node-negative gastrointestinal cancer, even after curative resection of an early cancer, some patients die of metastasis and recurrence^[1]. Metastasis and recurrence result from dissemination of cancer cells. Micrometastasis has been proposed by many investigators. Usually it is through blood and lymphatic vessels and no more than 2 mm in diameter^[2]. Detection of occult metastatic cells is useful for prognosis, prediction of recurrence, and adjustment of therapies.

Among many prognostic factors, lymph node metastasis is one of the most useful indicators for patients with gastric and colorectal carcinoma^[3-7]. Metastasis is usually detected by conventional histological examination, but many negative lymph nodes have micrometastasis on re-examination by serial sectioning and immunohistochemical assay^[8-11]. Serial sectioning and immunohistochemical staining certainly increase the yield of occult metastasis, however, it seems to be time-consuming and labor intensive. These methods have not been performed routinely in most hospitals. To overcome this drawback, diagnostic procedures for the detection of micrometastasis at the genetic level have developed rapidly, such as RT-PCR^[12-14]. RT-PCR can detect genes that are exclusively expressed in carcinoma cells but not in normal lymph nodes or bone marrow. It is a highly sensitive and specific method. It was reported that by RT-PCR it was possible to detect one cancer cell from among 10^4 to 10^6 normal appearing lymph node cells. Lymph node occult metastasis of gastrointestinal cancer indicated by K19 mRNA expression can be considered as confirmation of the presence of metastasis. The current study was designed to investigate the value and prospect of RT-PCR in detecting micrometastasis in regional lymph nodes of gastric cancer by examination of K19 mRNA expression.

MATERIALS AND METHODS

Tissue samples

The 30 tumor specimens and 126 lymph nodes were obtained through radical resection of gastric carcinoma of patients from the Department of General Surgery, First Hospital, Jilin city from 2001 to 2002, and tumor specimens were confirmed by pathology. The specimens

were processed immediately after the resection: tumor tissues were obtained; lymph nodes were peeled off carefully. Fat tissue and blood were wiped off, lymph nodes were cut into two halves by clean bistouries, and sterilized physiological saline was used for rinsing to prevent the contamination of tumor cells. One half of a lymph node was fixed by formaldehyde; the other half was immersed into liquid nitrogen, and then preserved in a -70°C freezer till the next day for RNA extraction. Meanwhile, lymph nodes from 8 non-tumor patients were used as negative control.

Reagents

The reagents included TRI reagent (GIBCO), AMV, Taq enzyme, DNTPs and Rnasin (Promega), Marker and Olig (dt) (TaKaRa); the rest of the reagents were all homemade provided by local suppliers.

Methods

Primer design and synthesis: Primer design of CK19 and β -actin was based on previous methods^[15] with some modifications. CK19 primer is: 5'-AGGTGGATTCCGCTCCGGGCA-3', 5'-ATCTTCCTGTCCCTCGAGCA-3'. The amplification fragment of the primer (Wubo Gene Corp., Beijing) was 460 bp. β -actin primer is: 5'-GTGGGGCCCCAGGCACCA-3', 5'-CTTCCTAATGTCACGCACGATTTC-3'; the amplification fragment of the primer (Dinguo Bio Corp., Beijing) was 540 bp. It was used as an internal control to determine that the RNA did not decompose.

RNA extraction: TRIzol was used to extract total RNA. The absorbency (A) was tested. Then gel electrophoresis was performed to identify its components.

Reverse transcription (cRNA synthesis): Reverse transcription system was 50 μL , containing RNA 4 μL , Oligo(dt) 2 μL , 5 \times Buffer 10 μL , dNTPs 4 μL , Rnasin 1 μL , AMV 4 μL and DEPC- H_2O_2 5 μL incubated for 60 min at 42°C , to acquire cRNA.

PCR reaction: The reaction system was 100 μL , containing cDNA 25 μL , 10 \times Buffer 10 μL , dNTPs 8 μL , K19 primer 2 μL , β -actin primer 0.5 μL , Taq enzyme 1 μL , MgCl_2 6 μL , DEPC- H_2O_2 47.5 μL . The cycle parameters of the reaction system were 94°C for 45 s, 55°C for 45 s, 72°C for 1 min, 35 cycles and extension for 10 min at 72°C .

PCR product analysis: PCR products were electrophoresed on 2% agarose gel, EB stained, and observed with ultraviolet light and photographed. The results were compared with pathological result.

Statistical analysis

We used t-test to compare between the histopathological results and RT-PCR results. $P < 0.05$ was taken as significant.

RESULTS

Comparison between histopathological result and K19 mRNA RT-PCR in detecting micrometastasis in regional lymph nodes

In 126 lymph nodes, 26 were positive in both routine

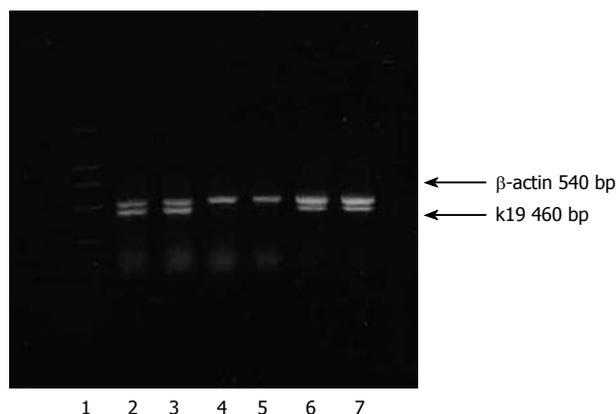


Figure 1 Representative results of RT-PCR. Lane 1: Marker; Lane 2: Tumor tissue sample; Lane 3: Negative lymph node by routine histopathology (K19 positive); Lane 4: Negative lymph node by routine histopathology (K19 negative); Lane 5: Normal lymph node of the non-tumor cases; Lanes 6, 7: Positive lymph node by routine histopathology.

histopathological testing and RT-PCR, K19 mRNA was expressed in 42 lymph nodes by RT-PCR amplification. It showed that there was metastasis in 16 lymph nodes which could not be found by histology examination. Of the regional lymph nodes, 20.6% were positive in histology, while 33.3% were positive in K19 mRNA by RT-PCR; and there were none that were positive for histology and negative by RT-PCR. In 30 cases of gastric tumor, 3 were positive in regional lymph nodes by RT-PCR while pathology showed no metastasis.

Specificity of RT-PCR amplification

All the tumor tissues and metastatic lymph nodes showed amplification fragments of 460 bp and 540 bp after RT-PCR amplification of K19 mRNA and β -actin, while in the lymph nodes of 8 non-tumor cases it showed only specific amplification fragments of 540 bp, indicating no K19 mRNA amplification product was expressed. Thus this system had superior amplification specificity (Figure 1).

DISCUSSION

There has been no uniform criterion for micrometastasis. In general, a focus not larger than 2 mm is called micrometastasis, which can not be easily found by routine method, whereas RT-PCR could increase the detection rate greatly. Zheng *et al*^[16] suggest selecting an ideal marker gene of the tumor as a histology specific marker. Keratin 19 is one of the histology markers, which is highly specific and only expressed in tumor tissue and tumor-originating normal tissue, but not expressed in normal mesenchymal tissues like lymph nodes^[17-20].

In this study, K19 mRNA was expressed in both tumor tissue and metastatic lymph nodes, but not in non-tumor cases. It indicates that K19 mRNA is applicable to detect the micrometastasis in regional lymph nodes by RT-PCR amplification. Moreover, the results of our study suggest that RT-PCR is more sensitive than routine histopathology in detecting micrometastasis and K19 can be a sensitive index for detecting metastasis in regional lymph nodes,

which accords with Liu's report^[21].

Because RT-PCR is the method that is sensitive and has great capability to amplify, sometimes there can be a pseudopositive, and the main reason could be that the lymph nodes are contaminated by tumor cells and normal epithelial cells and there is cross-contamination of byproduct during RT-PCR amplification. In the experiment, as the internal control, β -actin ensured reliability and the result showed that no K19 mRNA was expressed in any of the non-tumor cases under the same amplification condition, indicating that there was no pseudopositive in the RT-PCR amplification system. K19 mRNA RT-PCR has a high sensitivity and specificity in detecting micrometastasis in regional lymph nodes of gastric cancer and can detect the subtle metastasis which cannot be found by routine histology. This is of great clinical significance. According to the present staging criterion for gastroenteric cancer, the positive result will lead to change in the staging of tumor and alteration in therapy and prognosis judgment. Ye^[22] and Yan^[23] concluded that compared with lymph node-negative cases, K19 mRNA RT-PCR has obvious prognostic value on recurrence and survival time for the patients after the operation, even if there is a single metastasised tumor cell in the lymph node. Thus, the resection for early and intermediately staged patients should be as radical as possible^[24-25], so as not to miss the micrometastasis in lymph nodes and to reduce the recurrence; and the adjuvant therapy and follow-up should be enhanced as well.

The development of the subtle tumor cells in the lymph nodes depends on the immunity of the human body and other factors, which is possible but does not develop into an obvious metastasis. At present, there are still some questions left unanswered about detecting the micrometastasis in regional lymph nodes: one is that pseudopositives are possible because of its great capability of amplification; the other is how to choose a more specific tumor marker. Before the advent of serial analysis of micrometastasis of tumor cells, tumor cells were separated by an immunomagnetic method and extracted, which was thought to be the most attractive technique^[26]. Okadda^[27] put forward that a multiple-marked RT-PCR has a better effect on detecting micrometastasis in lymph nodes, and researchers are trying to find more sensitive and specific tumor markers including combinations of multiple-markers.

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Corrosive injury to upper gastrointestinal tract: Still a major surgical dilemma

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Abstract

In the developed and developing countries, corrosive injury to the gastrointestinal system as a consequence of either accidental ingestion or as a result of self-harm has become a less common phenomenon compared to decades ago. This could partly be attributed to the tighter legislation imposed by the government in these countries on detergents and other corrosive products and general public awareness. Most busy upper gastrointestinal surgical units in these countries, especially in the developed countries will only encounter a small number of cases per year. Up to date knowledge on the best management approach is lacking. In this article, we present our experience of two contrasting cases of corrosive injury to the upper gastrointestinal tract in our thoracic unit in the last 2 years and an up-to-date Medline literature search has been carried out to highlight the areas of controversies in the management of corrosive injuries of the upper gastrointestinal tract. We concluded that the main principle in managing such patients requires a good understanding of the pathophysiology of corrosive injury in order to plan both acute and future management. Each patient must be evaluated individually as the clinical picture varies widely. Signs and symptoms alone are an unreliable guide to injury.

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Key words: Acid; Alkali; Oesophageal stricture; Endoscopy; Steroids; Oesophageal and gastric carcinoma

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INTRODUCTION

In the developed and developing countries, corrosive injury to the gastrointestinal system as a consequence of either accidental ingestion or as a result of self-harm has become less of a common phenomenon. This could partly be attributed to the tighter legislation imposed by the government on detergents and other corrosive products and general public awareness^[1,2]. Most busy upper gastrointestinal (GIT) surgical units, especially in the developed countries will only encounter a small number of cases per year. Up to date knowledge on the best management approach may therefore be lacking.

We present two contrasting cases of corrosive injury to the upper GIT, which presented to our thoracic unit in the last 2 years to highlight the contrasting aspects of chemical burn injury to the upper GIT. A Medline search has been carried out to extract relevant articles to enable us to perform a literature review to discuss the areas of controversies in the management of corrosive injuries of the upper GIT.

CASE REPORTS

Case 1

A 22-year-old male with learning disability attended the Casualty Department following accidental ingestion of a cupful of 30% caustic soda and had vomited immediately after it. On presentation, his voice was hoarse. He was also short of breath and drooling his saliva. On examination, he had a red, swollen tongue and his oropharynx was oedematous and inflamed. He was intubated to secure his airway and transferred to the intensive care unit. Other supportive treatments received included intravenous proton pump inhibitor (PPI) and total parental nutrition (TPN). He was extubated 2 d later. Early oesophagogastrosocopy (Figure 1) revealed generally inflamed oropharynx and Savary grade 3 oesophagitis from 20 cm. Examination beyond this point was not attempted. Barium meal (Figure 2) carried out two weeks later showed a long stricture segment from just distal to the hypopharynx to the oesophago-gastric junction. The patient did not receive steroid therapy during any stage of his treatment. He was successfully managed with repeated progressively time spaced dilatation using a guide wire under fluoroscopy. He currently attends Day Procedure Unit every 6-12 wk for oesophageal dilatation.

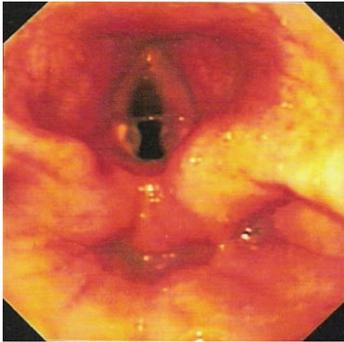


Figure 1 Endoscopic picture showing gross laryngeal edema with inflamed adjacent structures (case 1).

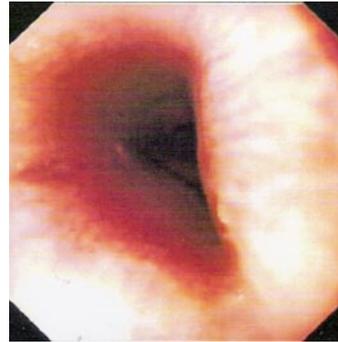


Figure 3 Oesophagogastric dissociation (OGD) of the patient after ingestion of battery acid showing circumferential burn to the lower oesophagus (case 2).



Figure 2 Barium meal showing a long stricture segment from just distal to the hypopharynx to the oesophago-gastric junction (case 1).



Figure 4 Barium study of patient showing partial gastric outlet obstruction with marked gastric dilatation with an irregular stricture of the pylorus and proximal duodenum (case 2).

Case 2

A 33-year-old male with a history of overdose and assaults presented to the Casualty Department with hoarseness and stridor following ingestion of about 40 mL of battery acid (hydrochloric acid) secondary to deliberate self-harm. He was intubated to secure his airway and transferred to ITU. A CT scan of his chest and abdomen showed thickened distal oesophagus and stomach, small bilateral pleural effusion and no obvious sign of perforation. Early endoscopy showed inflammation and ulceration of the pharynx and oesophagus with contact bleeding and circumferential ulceration of the oesophageal mucosa at 25 cm (Figure 3). His stomach was filled with blood. The immediate supportive treatments included intravenous PPI, TPN for nutrition, steroids and broad-spectrum antibiotics because of gross laryngeal oedema and positive blood culture. He improved on conservative management and following extubation was allowed oral feeding. Endoscopy was repeated 10 d later and it showed small ulcers at the level of the vocal cords. Upper oesophagus was relatively spared. A tight cricopharyngeus was noted and his lower oesophagus showed a circumferential burn with slough. Similar findings were noted on the mid-body of the stomach and the antrum but the duodenum was spared.

He was discharged home three weeks later only to be readmitted a week and a half post-discharge with symptoms and signs of gastric outlet obstruction. Endoscopy at this stage showed a normal oesophagus with an ulcerated and scarred gastric pylorus. The endoscope failed to advance beyond this point. These findings were confirmed on a dilute barium swallow (Figure 4). Roux-en-Y gastrojejunostomy was performed subsequently

in order to bypass the stricture. He remained well post-operatively.

DISCUSSION

There are a vast variety of chemicals commonly available in a modern western household that can be ingested either inadvertently or intentionally. Failure to recognize the seriousness of the accident and to provide adequate therapy could result in serious morbidity and mortality. Children account for more than 80% of accidental corrosive ingestion but ingestion in adult is more often of suicidal intent, and, therefore, tend to be more serious^[3]. The mortality rate is between 10% to 20% and rises to 78% in cases of attempted suicide^[4]. The extent of the injury depends on the type of agent, its concentration, quantity and physical state, the duration of exposure and the presence of food particles in the stomach^[5-7].

Pathophysiology

The dichotomy of oesophageal versus gastric injury in cases of acid and alkali ingestion has long been recognized by surgeons and gastroenterologists^[8]. Whilst acid is said to “lick the oesophagus and bite the pyloric antrum”, alkaline tends to cause a more uniformly severe mucosal injury to the oesophagus^[3,6,9]. Although acid injury is usually limited to the stomach, 6%-20% of patients have other associated oesophageal and small intestinal injuries^[6]. Our two cases clearly illustrated this with the caustic injury in case 1 causing extensive oesophageal injury whilst the acid resulting in gastric injury and sparing the oesophagus.

Acid injuries cause “coagulation necrosis” on tissue contact; the coagulum formed hinders any further tissue

penetration^[3,4,9,10]. On the other hand, caustic injuries induce “liquefaction necrosis”, a process that leads to the dissolution of protein and collagen, saponification of fats, dehydration of tissues and thrombosis of blood vessels, resulting in deeper tissue injury^[3,4,9,10]. Zargar *et al* noted that acute gastric injury was present in 85.4% of their patients who had ingested acid, involving mainly the distal half of the stomach with 44.4% having late complications in the form of pyloric or antral stenosis and linitis plastica-like deformity^[11]. The relative sparing of the duodenum is thought to be due to pyloric spasm induced by the irritant acid in the antrum and the alkaline pH of the duodenum^[11]. Our patient who ingested battery acid developed partial gastric outlet obstruction. Contrast study performed documented duodenal rigidity and lack of normal mucosal pattern. However, the distinction between the expected sites of gastrointestinal injury following acid versus alkali ingestion is not always clear.

Burn classification

Injuries secondary to chemical burns of the upper gastrointestinal tract are classified in similar fashion to thermal burn of the skin. They are classified into three degrees based only on the extent and severity of the superficial lesions^[12]. An appreciation of the depth of the involvement may improve our treatment, but at present, no definite measurements of the depth can be made, and the grading at best is subjective. Endoscopic ultrasound may provide an answer. A third-degree burn can easily be mistaken for a second-degree burn^[9].

Oropharyngeal burns and clinical symptoms have a low predictive value for severity of oesophageal injury^[13,14]. Haller *et al* observed that 70% of their patients with oropharyngeal burns did not have significant damage to the oesophagus^[15]. Both our patients had severe oropharyngeal burns requiring immediate intubation in order to protect their airway but only one developed severe oesophageal injury.

Early versus late endoscopy

Early endoscopy is regarded as the most appropriate measure based on which clinical decisions are made in people who have ingested corrosive substances^[3,8,12,16-18]. The majority of physicians and surgeons now favour early endoscopy. Nevertheless, early endoscopy in the hands of a less-experienced endoscopist could be hazardous^[2,10]. It is important to minimize force and air insufflation when passing the endoscope in this group of patients^[10]. The risk of oesophageal perforation is low if the procedure is performed under general anaesthesia and the endoscope is passed to the first burned area but not beyond it^[16]. Hawkins *et al* recommend diagnostic oesophagoscopy under general anaesthesia within the first 36 h of corrosive ingestion. In severe oropharyngeal burns, endoscopy may be deferred up to seven days to allow the acute oedema to subside, thereby reducing the risk of airway complication^[8]. Zargar *et al* performed endoscopies on 88 patients within 96 h following corrosive ingestion and found no complications related to the procedure^[19]. Others have also documented the safety of early endoscopy^[3]. Both our

patients had endoscopy carried out within 72 h following corrosive ingestion. Early endoscopy is essential in the continuing management of patients with corrosive injury as it affords an opportunity to verify directly the healing state of the mucosa and may be of value in predicting which patients require further early intervention^[1,2,8,19,20].

Endoscopy is not without its limitations^[1]. It is difficult to assess the depth of any burn with absolute certainty by observing superficial epithelial necrosis^[2]. If a severe burn is encountered in the upper third of the oesophagus, the scope is not passed beyond this point. In this case, it will be difficult to ascertain the degree of injury to the rest of the oesophagus^[3]. The area of burn may not be visualised, thus delaying the diagnosis^[2]. Others have attempted the use of endoscopic ultrasound to improve the accuracy of diagnosis. However, Chiu *et al* did not find concomitant use of endoscopic ultrasound (EUS) useful in improving the accuracy of predicting early or late complication of stricture^[21].

Manometric study

Genc and Mutaf investigated the use of manometric studies and suggested that it could give important data about the severity of the initial oesophageal injury^[22]. Dantas *et al* showed that a majority of their patients with caustic oesophageal injury exhibit alterations in oesophageal motility, ranging from low amplitude non-peristaltic contractions to some degree of alteration of lower oesophageal sphincter pressure^[5]. Thus, it could play a role in determining the prognosis. PPI is generally prescribed on the basis of associated reflux. This data provide supporting evidence for its use.

Complications of corrosive ingestion

Severe complications, often life threatening are common following corrosive injury to the upper gastrointestinal tract. These include tracheobronchial fistula in 3%, severe haemorrhage secondary to gastric involvement, aortoenteric fistula or gastrocolic fistula, strictures and perforation in 10% of cases^[3,5,13,20]. Stricture formation, by far, remains the main long-term complication of this injury. Over 90% of patients with third-degree burns go on to develop stricture and 15%-30% if they have second-degree burns^[3]. Mamede *et al* observed an 89.3% incidence of oesophagitis in their 37-year historical series; 72.6% of the cases involved progression to stenosis and 1% died during acute phase^[7]. A lumen >10 mm in diameter is thought not to impede normal life and should be left alone^[13].

Early use of steroids and antibiotic: Prevention of stricture formation

Corticosteroids inhibit the transcription of certain matrix protein genes, procollagen, fibronectin, TGF- β and many cytokines. They also reduce the synthesis of α 2-macroglobulin, an inhibitor of collagenase activity^[23]. Animal experiments have shown that if antibiotics and steroids are given early following ingestion of a corrosive substance, the likelihood of stricture formation is reduced^[2,24]. Bautista *et al* found dexamethasone more

effective than prednisolone in preventing stricture formation following experimental oesophageal burns^[24]. Mamade *et al* concluded from their clinical experience of 239 cases over 38 years that lower doses of steroid have little effect on the prophylaxis of stricture^[25]. Higher doses only seem to contribute to the onset of complications such as increased vulnerability to infection and gastrointestinal bleeding^[16,25]. Several authors have found corticosteroids ineffective in preventing oesophageal stricture^[10,12,14,16,18]. This has also been shown in a more recent randomised controlled clinical trial in children.

Intra-lesional corticosteroid therapy has shown beneficial effects for refractory oesophageal strictures caused by corrosive burns. A report by Kochhar *et al* concluded that patients treated in this way experience a longer dilatation-free interval, thus requiring fewer dilations^[26]. However, the number of patients involved in the study is small.

In our two contrasting cases, steroid was given to the patient with acid burns for the first 24 h in view of the severity of laryngeal oedema at presentation to avoid casualty. In another patient who suffered from caustic burns, steroid was not given because the literature suggests that the complication risk outweighs the efficacy in preventing stricture formation.

To date, there is no convincing evidence supporting the use of antibiotics in reducing stricture formation^[18]. An animal study has shown that it could decrease infection in steroid treated burns^[3]. Kirsh *et al* recommended the use of antibiotics for 7 d to 2 wk as a means to both decrease the risk of pulmonary infection and bacterial invasion through the injured oesophagus into the mediastinum^[2]. Our general consensus when treating a patient with such injury is that antibiotic treatment should only be commenced when the patient is treated with steroids or there are signs of infection with source of infection and infecting organism identified. Prophylactic use of antibiotics without steroid treatment is unjustified^[18].

Routine use of nasogastric (NG) tube

Mamede *et al* reported a significant lower incidence of stricture formation with routine use of NG tube for 15 d following the injury^[25]. However the NG tube could not act as an oesophageal 'mould' because one could expect the stenosing effect to continue longer than 15 d. Wijburg *et al* also reported a decline in stricture formation in a patient with long-term nasogastric tube placement^[27].

However, contrasting results were obtained from other literatures stating that long-term indwelling nasogastric insertion is known to cause long strictures of the oesophagus even in patients without oesophageal burns^[3,18]. We do not advocate the use of a NG tube as we have experienced a number of patients who developed complex stricture following nasogastric insertion. Furthermore, the presence of a NG tube will aggravate reflux by making the lower oesophageal sphincter incompetent. We used TPN in both patients and would have proceeded to feeding jejunostomy if oral feeding was not soon established.

Experimental studies to prevent stenosis

In a recent experimental study, cytokines have been used

successfully in preventing stricture formation by Berthet and colleagues^[20]. The theory was based on the rationale of the inflammatory process and cascade of events. Epidermal growth factor (EGF) was used because of its properties of fibroblast stimulation and improvement of local vascular conditions. Interferon- γ (IFN- γ) was also used to reduce fibrosis as it inhibits collagen I and III formation and fibronectin synthesis^[20]. Hydroxyproline was used as an indirect measurement of collagen production as it is the ultimate product of collagen degradation^[20]. Stenosis was not observed in treated animals^[20]. There was a lower level of hydroxyproline in combined treatment compared to EGF alone^[20].

Kaygusuz *et al* investigated the effect of interferon- α -2b and octreotide in the treatment of corrosive burns of the oesophagus^[28]. A histopathological examination of the exposed oesophagus demonstrated that octreotide and interferon- α -2b distinctively depressed the fibrotic activity in the second phase of wound healing that occurred in the oesophageal wall after a corrosive burn^[28]. Gunel *et al* showed in their animal experiment that treatment with an antioxidant, such as vitamin E and methylprednisolone decreased tissue hydroxyproline and thus, inhibiting new collagen synthesis and stricture formation following corrosive injury^[29]. However, all these studies are only carried out on animals and these treatments have not been tested on humans.

Management

The acute management is based on the acute trauma life support (ATLS) guidelines for burn injury. This includes securing the airway, pain relief and attending to adequate intravenous fluid replacement. Tracheostomy may be necessary in cases of severe laryngeal oedema, whereby tracheal intubation fails and there is a danger of completely closing over of the airway due to the edema^[2,30]. The aim of treatment at this stage is to stabilize vital parameters. The patient is kept strictly nil by mouth in acute phase. A plain chest radiograph is advisable and might reveal signs of perforation, i.e. pneumomediastinum and free air under the diaphragm^[3,19,28,30]. However the sensitivity is low and if perforation is suspected, diluted barium swallow should be carried out. It is crucial that the attending medical officers are aware of the severity of such injury and able to identify life-threatening complications associated with the injury. The use of antidote such as water or milk does not seem to prevent stenosis^[25]. Endoscopy is the diagnostic procedure of choice in the absence of known perforation^[3]. Patients with perforation require immediate surgery^[3]. Gastric acid suppression with PPIs and H₂-antagonists are often used in corrosive burn injury as oesophagitis and gastritis are common and patients have been kept fasting^[18]. This treatment has been employed in both our patients in order to suppress gastric acid production and to prevent stress ulcers in the stomach.

Our first patient with the alkaline burns of the gastrointestinal tract who later developed oesophageal strictures was managed with frequently repeated dilatation. Hawkins *et al* reported a relatively high success rate with dilatation^[16]. Dilatation could be antegrade or retrograde or a combination of

both^[1]. Early dilatation is not recommended due to associated high incidence of perforation and associated morbidity^[3]. Most authors advised waiting 3 to 6 wk after the initial injury before attempting oesophageal dilatation^[3,30]. Overall, oesophageal dilatation has proved to give good results in short strictures but might be dangerous for long and narrow oesophageal strictures^[14,20]. Complex strictures are refractory to dilation therapy and fluoroscopic guidance has a valuable role in managing these types of strictures^[23]. Repeat dilation sessions are needed in most cases with a goal of achieving a luminal diameter of 12 mm or larger in order to alleviate symptoms of solid dysphagia^[23]. We suspect that the strategy of intense PPI therapy and repeated dilatation will reduce the number of impassable stricture that otherwise would have required oesophageal resection and reconstructive surgery.

The second patient with acid burns of the gastrointestinal tract developed gastric outlet obstruction within 3 wk of injury. The use of a steroid is of questionable value, and overall evidence from the literatures is not in favour of routine use. This is because it could mask the clinical signs of free perforation and infection^[12,13,16]. Therefore, its use is limited mainly to patients with severe laryngeal oedema. Antibiotics have been used in this case for the treatment of an obvious chest infection. Gastric outlet obstruction has been found in association with oesophageal stricture in the region in 20% of cases^[17]. In some cases gastric outlet obstruction can yield balloon dilatation but our patient required surgical bypass because of the complex nature of his stenosis. Alternative surgical reconstruction would be hemigastrectomy and resection of the first part of the duodenum with Bilroth I reconstruction. At the time of presentation, our patient was unfit for such a major operation.

Understanding the pathophysiology of corrosive injury is important in planning both acute and on-going management. Scar retraction begins as early as the end of the second week and lasts for 6 mo. Six to twelve months is considered the average time before full fibrosis is achieved after the injury^[31]. Oesophagectomy carried out too early prior to the scar tissue maturation might increase the risk of anastomotic stenosis^[32]. Han *et al* advocate delaying major reconstructive surgery in patients with caustic burns for at least 6 mo from time of injury provided that emergency surgery is not indicated^[32]. Emergency oesophagectomy plus exteriorisation or immediate reconstruction is however indicated in cases of perforation and contamination of the mediastinum^[9].

Risk of carcinoma

The association of lye stricture and carcinoma of the oesophagus has been known since 1896^[2]. Kiviranta believed that the incidence of oesophageal cancer among victims of lye stricture is at least 1000 times greater than that in the normal population^[33]. The interval between lye ingestion and the development of carcinoma ranges between 25 to 40 years. However, this risk may be overestimated. Marchand did not encounter a single case in 135 patients with caustic strictures of the oesophagus over a period of 6 years^[34]. Carver and colleague had 2 patients out of 233 patients with lye strictures over a period of 25

years^[35]. Mamede and colleague found 4 (1.6%) out of the 239 patients from their 37-year historical series developed oesophageal cancer after caustic soda ingestion^[7]. In these cases, operative risk may exceed the potential risk of cancer.

The risk of gastric cancer is less known^[8]. Gray and Holmes first reported in 1948 findings of squamous metaplasia in the stomach of a patient who had ingested acid^[56]. Similar findings were subsequently reported by O'Donnell and colleagues^[37] and later by Eaton and Tennekoon^[38]. Some surgeons are more aggressive in resection of the involved stomach because of the danger of subsequent gastric metaplasia^[6,9]. The predisposition to cancer justifies regular follow-up and surveillance endoscopy. However, the patient should be warned of the cumulative dangers of other risk factors for oesophageal cancer, such as alcohol abuse and smoking^[30].

CONCLUSION

The literature on treatment of patients with corrosive injuries to the upper gastrointestinal tract is both controversial and inconclusive. The main principle in managing such patients is that each patient must be evaluated individually as the clinical picture varies widely. Signs and symptoms alone are an unreliable guide to injury. Both the acute and the chronic phases of the clinical presentation require different management. Psychiatric support is sometimes needed during both the acute and chronic phases. The general consensus is that the initial treatment is supportive; ensuring the airway is patent and to establish haemodynamic stability. Early endoscopy has a crucial role in both diagnosing the severity of the injury, as well as, in managing the patient. Total parenteral nutrition is a useful adjunct. Operation is generally reserved for patients who have ingested large amounts of corrosive substance and in whom tissue necrosis is highly likely. Extensive necrosis noted on endoscopy and patients with evidence of perforation are indications for immediate surgical intervention. As for intractable oesophageal strictures when dilatation is dangerous or impossible, surgical intervention may be unavoidable. However, this must be balanced against the mortality and sometimes considerable morbidity following surgery^[2,30]. Follow up endoscopy should be carried out within 6 wk following discharge from the hospital^[30]. Diligent follow-up is advised to ensure patient has satisfactory gastrointestinal function restored and to correct late onset complications.

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Enteric neuropathology of congenital intestinal obstruction: A case report

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One year after the latest surgery the patient tolerated oral feeding and did well, suggesting that congenital (partial) mechanical obstruction of the small bowel in humans can evoke progressive adaptive changes of the ENS which are similar to those found in animal models of intestinal mechanical occlusion. Such ENS changes mimic neuronal abnormalities observed in intestinal pseudo-obstruction.

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Key words: Enteric neuropathy; Chronic intestinal pseudo-obstruction; Congenital intestinal obstruction; Ladd's band; Enteric nervous system

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Abstract

Experimental evidence indicates that chronic mechanical sub-occlusion of the intestine may damage the enteric nervous system (ENS), although data in humans are lacking. We here describe the first case of enteric degenerative neuropathy related to a congenital obstruction of the gut. A 3-year and 9-mo old girl began to complain of vomiting, abdominal distension, constipation with air-fluid levels at plane abdominal radiology.

Her subsequent medical history was characterized by 3 operations: the first showed dilated duodeno-jejunal loops in the absence of occlusive lesions; the second (2 years later) was performed to obtain full-thickness biopsies of the dilated intestinal loops and revealed hyperganglionosis at histopathology; the third (9 years after the hyperganglionosis was identified) disclosed a Ladd's band which was removed and the associated gut malrotation was corrected. Repeated intraoperative full-thickness biopsies showed enteric degenerative neuropathy along with reduced interstitial cells of Cajal network in dilated loops above the obstruction and a normal neuromuscular layer below the Ladd's band.

INTRODUCTION

The enteric nervous system (ENS), the third component of the autonomic nervous system, plays a crucial role in the control of gastrointestinal functions, including motility, secretion, absorption, blood flow, mucosal growth and aspects of the local immune system^[1]. Hence, any condition altering the integrity of the ENS is responsible for a wide spectrum of disorders affecting the gastrointestinal tract. Abnormalities of the ENS (also referred to as enteric neuropathies) may be secondary to a variety of inflammatory, infectious, metabolic and neurological diseases, or they can be labelled idiopathic when no causes can be found^[2-6]. The most common clinical manifestation related to an underlying enteric neuropathy (either idiopathic or secondary) is a severe functional impairment of gastrointestinal motility as that identifiable in patients with chronic intestinal pseudo-obstruction (CIPO)^[4,7,8]. The diagnosis of CIPO is based on the exclusion of any mechanical lesion occluding the

gut lumen^[4,7,8]. On the other hand, in animals chronic incomplete mechanical occlusion of the intestine may induce ENS damage^[5]. The effects of chronic obstruction on the human ENS are still unknown.

Herein we describe the case of a girl in whom an initial diagnostic work-up suggested a diagnosis of CIPO with an underlying neuropathy characterized by hyperganglionosis of the ENS, whereas a laparotomy about 10 years later showed a chronic congenital obstruction of the small bowel due to a Ladd's band. Full-thickness gut biopsies, taken during this last laparotomy, showed an enteric degenerative neuropathy in the bowel loops located above the obstruction. Because the correct pathology remained undiagnosed for a long time, this case can be considered a human model of mechanical partial obstruction of the gut. Specifically the case indicates that a long-standing ("chronic") intestinal sub-occlusion can evoke progressive changes of the ENS similar to those found in animal models, and that the observed ENS changes mimic abnormalities often found in CIPO related to idiopathic neuropathies.

CASE REPORT

G. S. was a full term baby girl weighing 2040 g at delivery whose clinical history was unremarkable until 3 years and 9 mo of age when she began to complain of post-prandial vomiting (often bilious) and constipation. Clinical examination revealed abdominal distension, dehydration and decreased bowel sounds. Systemic and neurologic diseases, as well as infections and malabsorption syndromes were excluded by physical examination and blood tests. X-ray of the small bowel showed mild gaseous distension of the proximal small bowel loops with air-fluid levels although a CT scan failed to reveal any mechanical obstruction. Due to the severity of the clinical picture and the marked small bowel distension, the patient underwent laparoscopy showing duodeno-jejunal dilatation with no obvious mechanical cause. The patient was discharged with a diagnosis of intestinal pseudo-obstruction and treated with prokinetics and a low-fiber diet.

Subsequently, she had recurrent episodes of vomiting, abdominal pain, early satiety and fullness. Because of the persistence of severe dyspeptic symptoms, at 5 years and 7 mo of age the patient was referred to a tertiary center for pediatric gastroenterology where, based on the clinical history and previous examinations, a decision to undertake an explorative laparotomy was reached. The intra-operative evaluation showed a marked degree of duodeno-jejunal dilatation but no mechanical obstruction.

Analysis of full-thickness biopsies taken from the dilated jejunal loop revealed enlarged myenteric and submucosal neurons whose number was increased as compared to sex-age-matched normal controls^[9]. A further important feature was the increased density of the nerve fibers in the lamina propria and submucosa. Based on the histopathology suggesting a case of hyperganglionosis, the diagnosis of CIPO was confirmed and the patient received several courses of prokinetics, metronidazole and high-caloric liquid diet supplementation.

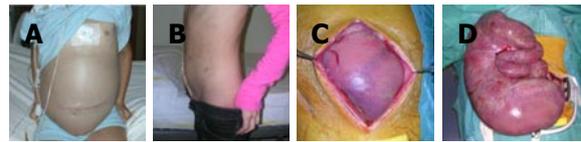


Figure 1 Representative pictures showing clinical (A and B) and laparoscopic views (C and D) observed in this case. Note (A) the hugely distended abdomen and (C and D) abnormally dilated loops located proximally to the removed Ladd's band (not visible in these examples) at laparotomy. B illustrates the considerable deflation of the abdomen following surgery.

During a subsequent recovery the patient underwent an upper gastro-intestinal manometry which showed a pattern indicative of neurogenic-type CIPO. Due to the progressive worsening of her clinical status, at 14 years and 2 mo of age, the patient was referred to our center in Bologna. Physical examination showed a highly distended (Figure 1A) and painful abdomen, with absence of peristaltic sounds. An X-ray examination showed multiple air-fluid levels in the upper abdomen with a striking elevation of the diaphragm. In order to achieve bowel decompression, she underwent surgery. At laparotomy a Ladd's band was detected and removed with correction of the associated malrotation. Bowel loops with marked dilatation (Figures 1C and 1D) were resected and a protective ileostomy was created. Full-thickness biopsies were taken from the loops located proximally (dilated segments) and distally (macroscopically normal) to the Ladd's band.

Following surgery, the clinical course was uneventful with immediate deflation of the abdomen. One year after the last laparotomy the patient was healthy and tolerated oral feeding, her height and weight were markedly increased, and her quality of life was good (Figure 1B).

Immunohistochemical analysis

Full-thickness tissue specimens were processed for immunohistochemistry according to standard protocols commonly applied in our laboratory. Compared to controls (jejunal specimens collected from patients operated on for intestinal bleeding due to angiodysplasia; $n = 4$, 2 females, age range: 6-16 years) (Figure 2A) and the non-dilated segment (Figure 2B), the immunohistochemical evaluation of biopsies taken from the dilated loop showed evidence of intrinsic neuropathy of the gastrointestinal tract characterized by severe myenteric and submucosal neuron depletion (Figure 2C), as identified by the reduced number of neural elements labeled by the general marker neuron specific enolase (NSE) (purchased from DakoCytomation, Milan, Italy). Furthermore, analysis of several transmitters/neuromodulators of the ENS demonstrated a marked decrease of substance P, vasoactive intestinal polypeptide, calcitonin gene-related peptide (all these antibodies were kindly donated by Dr. C. Sternini and H. E. Wong, Center for Ulcer Research and Education/Digestive Diseases Center, UCLA School of Medicine, Los Angeles, CA) and nitric oxide synthase (purchased from BD Biosciences, San Jose, CA) in the myenteric and submucosal neurons and related processes of the dilated segment compared to

the non-dilated segment and controls. In order to explore the possible abnormalities of neuronal cell survival, we used a specific antibody against the product of B-cell lymphoma-2 (*BCL-2*) (DakoCytomation, Milan, Italy), a gene encoding a protein involved in cellular pathways of neuronal apoptosis^[10,11]. Compared to the controls (Figure 2D) and the non-dilated segment (Figure 2E), *BCL-2* immunoreactivity of the dilated segment was markedly reduced in myenteric (Figure 2F) and submucosal neurons and nerve processes. The interstitial cells of Cajal (ICC), visualized by an antibody against c-Kit (DakoCytomation, Milan, Italy), were markedly decreased in the dilated segment compared to the non-dilated segment and controls. Compared to the controls, the anti- α -smooth muscle actin antibody (DakoCytomation, Milan, Italy) showed an apparently normal muscular layer both in dilated and in non-dilated segments.

Transmission electron microscopy study

Full-thickness tissue specimens were also processed for electron microscopy according to standard protocols commonly applied in our laboratory.

Compared to the controls (Figure 2G) and the non-dilated segment (Figure 2H), electron microscopy analysis of myenteric (Figure 2I) and submucosal neurons of the dilated loop showed degenerative features characterized by nuclear chromatin clumping, shrinkage of the cell body and cytoplasmic vacuoles mostly deriving from the enlargement and matrix clearing of mitochondria.

DISCUSSION

The present case illustrates the occurrence of gut failure as a result of longstanding mechanical small bowel obstruction due to a Ladd's band, which has been unrecognized for more than 10 years despite extensive radiological investigation and 3 explorative laparotomies. A Ladd's band, which arises from the posterior abdominal peritoneum and extends from the liver to the colon (passing anteriorly to the duodenum), is responsible for gut malrotation^[12]. Symptomatic patients usually present either acutely with bowel obstruction and intestinal ischemia, or chronically with vague abdominal pain. Chronic symptoms can often make diagnosis difficult, as was the case in our patient. Although Ladd's band and associated gut malrotation are more common in the first 2 wk of life^[13,14], our case provides evidence that patients having this developmental abnormality may be found in childhood.

The clinical and laboratory features of this case have expanded our knowledge on the ENS neuropathology. First, chronic intestinal partial obstruction occurring as a result of a long-standing mechanical cause (Ladd's band) evokes changes similar to those found in animal models; second, enteric neuropathologic changes observed in the advanced stage of this case could not be differentiated from the neurodegenerative findings often detected in cases of CIPO.

In the current case, early tissue analysis of dilated bowel segments (i.e., from the region which was subsequently identified as proximal to the Ladd's band) showed hyperganglionosis of the ENS. These neuronal changes,

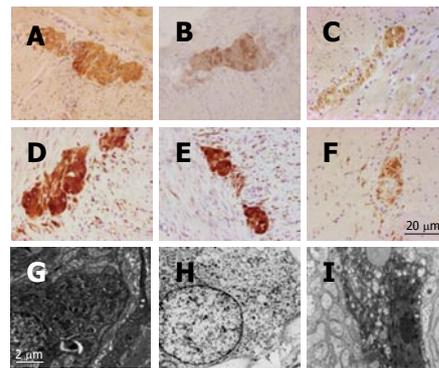


Figure 2 Representative examples illustrating the general neuronal marker NSE (A, B, C) and *BCL-2* (D, E, F) immunoreactivities in the neuromuscular layer of a control subject (A, D), in the non dilated loop distal to the congenital obstruction of the patient (B, E) and in the dilated loop proximal to the congenital obstruction of the patient (C, F). Note the marked reduction of NSE and *BCL-2* immunoreactivities in myenteric ganglion cells and nerve fibers targeting the muscular layer observed only in the dilated loop. Streptavidin biotin immunoperoxidase technique. Calibration bar (A-F): 20 μ m. Compared to controls (G) and the non-dilated segment (H), myenteric neurons of the present case (representative example in I) showed chromatin clumping, cell body shrinkage and cytoplasmic vacuolization. Uranyl acetate and lead citrate staining, transmission electron microscopy. Calibration bar (G, H, I): 2 μ m.

which were reported when the patient was 5 years old, may be considered the result of adaptive phenomena to a long-standing mechanical stimulus, such as obstruction limiting bowel propulsion. Several experimental models have been instrumental in supporting the concept that adaptive changes to the ENS occur proximal to a partially obstructed gut^[5,15-21]. These studies showed increased density and size in both myenteric^[16,18,19,21] and submucosal neurons^[19-21], along with neurochemical^[18,20] and cytoskeletal abnormalities of myenteric neurons^[21]. Recently, Galvez *et al*^[20] induced surgical stenosis (about 20% of the lumen) of rat sigmoid colon and reported changes in ENS structure after 6-12 wk. The molecular mechanisms involved in enteric neuroplasticity secondary to a partially obstructed bowel remain to be elucidated. Further, enteric neuronal changes, ICC (the pace-maker cells of the gut, which act in concert with neurons in governing gut motility) have been found to be reduced in experimental mechanical sub-occlusion of the small bowel as indicated by a decrease in c-Kit immunoreactivity and loss of functions attributed to ICC^[17,18]. In contrast, other data do not support the existence of ICC abnormalities (according to c-Kit immunostaining) in dilated gut segments of patients with Crohn's disease, a well-known cause of intestinal mechanical sub-occlusion^[22]. The possible explanations for the discrepancy between this and other studies remain to be defined.

About nine years after the initial observation of hyperganglionosis, the evaluation of new full-thickness biopsies from the dilated bowel above the stenosis showed evidence of a significant loss of both enteric neurons and ICC, which may explain the marked deterioration of the digestive function observed in our patient. Enteric neuron depletion is supported by the evidence of neurodegenerative changes detected by electron microscopy along with the reduced expression of the protein encoded by *BCL-2*,

a gene related to one of the intracellular pathways involved in the inhibition of programmed cell death^[2,3,10,11]. These findings indicate that neuronal cell loss may be due to apoptosis triggered by the persistent mechanical sub-occlusion of the gut. The neurodegenerative abnormalities observed in this phase of the clinical history of the patient may resemble ENS changes described both in humans^[23] and in an experimental model of small bowel atresia^[24]. In human intestinal atresia, Masumoto *et al*^[23] have shown hypoplasia of myenteric ganglia and marked reduction of both intramuscular nerve fibers and ICC in the dilated bowel segments above atresia. Similarly, in a chick embryo model of intestinal atresia, Schoenberg and Kluth^[24] have found an almost complete loss of both ganglionated plexuses in proximal dilated loops.

Taken together, enteric hyperganglionosis followed by degenerative changes can be considered the result of a bi-phasic adaptive process of the ENS in response to a persistent mechanical obstruction of the gut.

A further consideration which can be drawn from the present case concerns the accuracy of ENS pathology in patients with intestinal sub-occlusion. This case report clearly indicates that the enteric neuropathologic changes observed in different stages of a mechanical obstruction could not be distinguished from the neurodegenerative findings often detected in cases of CIPO. Neuropathology of CIPO includes a wide spectrum of ENS changes ranging from hyperganglionosis up to marked reduction of intramural (especially myenteric) neurons associated with swollen cell bodies, variable neurochemical abnormalities and fragmentation and loss of axons sometimes accompanied with proliferation of glial cells^[2-5]. If the mechanical obstruction remains unrecognized, as it did in our case, the ENS abnormalities mimic those identifiable in CIPO associated with an underlying neuropathy. Therefore, our case should be considered as a reminder that ENS changes observed in patients with suspected CIPO may be secondary in nature and should not necessarily be interpreted as definitive evidence for a primary neuropathy.

In conclusion, congenital (partial) mechanical obstruction of the upper small bowel leads to progressive adaptive/neuroplastic changes of the ENS similar to those described in experimental models of intestinal occlusion. Interestingly, such ENS abnormalities mimic those often observed in cases of CIPO.

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CASE REPORT

Unusual gastric and pancreatic metastatic renal cell carcinoma presentation 10 years after surgery and immunotherapy: A case report and a review of literature

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Abstract

Renal cell carcinoma (RCC) is the most common renal tumor, accounting for 2%-3% of all malignancies. Though RCC is known to spread hematogenously, isolated RCC metastasis to the stomach is a rare event. In this article, we describe the clinical course of a patient who developed a pancreatic recurrence of RCC and 1 year later a gastric recurrence of RCC treated 10 years ago with a resection and interleukin-2 (IL-2).

Accumulating evidence indicates that metastatic involvement of the pancreas and stomach should be suspected in any patient with a history of RCC who presents with gastrointestinal symptoms even 10 years after RCC resection and immunotherapy.

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Key words: Renal cell carcinoma; Stomach metastasis; Interleukin-2 treatment

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INTRODUCTION

Renal cell carcinoma (RCC) is the most common renal tumor, accounting for 2%-3% of all malignancies^[1]. It has

the highest incidence in the 6th decade and occurs twice as often in men compared to women^[2]. Several risk factors are associated with a high risk for RCC such as tobacco smoking, obesity, exposure to asbestos or chemicals, thiazide drug intake and urinary tract infections^[3]. RCC causes only few early warning signs. The symptoms are often non-specific and the triad of Virchow consisting of an abdominal mass together with flank pain, and macroscopic hematuria is nowadays rare^[4]. RCC spreads hematogenously and is known for its ubiquitous metastatic patterns. While pancreatic localization of recurrence is quite common^[4,5], the gastric localization of recurrences of this type of tumor is quite rare. Some authors have described a recurrence of RCC in the stomach but among them only a few are well documented^[6-14].

In this article, we describe a patient who developed a pancreatic recurrence of HCC and 1 year later a gastric recurrence of RCC treated 10 ago before with a resection and IL-2.

To our knowledge, this is the first report that describes the recurrence of metastatic RCC 10 years after a complete response to high-dose IL-2 therapy showing as a pancreatic metastasis and a year later again as a gastric metastasis.

CASE REPORT

This paper reports the history of a 68-year-old male patient. He underwent a right radical nephrectomy for RCC in 1990 followed by a high-dose IL-2 treatment. No other relevant disease was present in his past clinical history.

Ten years after the above-mentioned intervention he developed a disease recurrence involving the spleen and tail of the pancreas. He underwent a standard diagnostic work-up that included hemochemical determinations, then a splenectomy and distal pancreatectomy followed by a high-dose IL-2 therapy. No evidence of involvement of other abdominal organs was present.

One month after the last intervention, he visited the emergency room with a 10-d lasting history of polyuria and polydipsia. His physical examination was negative and hemochemical assays revealed the presence of hyperglycemia (494 mg/dL) and an increase of serum amylase (329 IU/L), while the hematocrit and differential blood counts were normal. The diagnosis was diabetes related to the recent distal pancreatectomy. He was treated with fast-acting insulin and discharged with a



Figure 1 Metastases of 1 and 3.5 cm in diameter as shown by chest X-ray.



Figure 2 An irregular mass (5 cm in diameter) corresponding to gastric metastasis, several para-aortic lymphadenopathies and liver metastatic nodules as shown by CT scan of the abdomen.

prescription of insulin. Eleven months later, he again visited the emergency room with a 3-d history of postural dizziness and weakness. He also reported melena the day before admission. The only other gastrointestinal symptom that could be elicited was early increasing satiety after meal during the previous year. No weight loss was present. Hematochemical determinations revealed anemia (hematocrit 17.7%, hemoglobin 56 g/L) with a rise in the count of platelets (579 000/ μ L). The level of glycemia at fasting was 453 mg/dL though the patient was following his therapy. No abnormal finding was present on physical examination. A nasogastric tube was placed and fresh blood in the stomach was revealed at aspiration. The patient immediately received intravenous fluids and 3 units of blood. His hyperglycemia was treated with fast-acting insulin. The patient underwent upper gastrointestinal endoscopy showing a multilobulated, polypoid, friable, and bleeding mass (5 cm in diameter) in the gastric fundus with a central bleeding ulcer. Epinephrine was injected into the lesion and the bleeding stopped. The patient underwent a further endoscopy 3 d later and a sample of the mass was taken for biopsy. Histopathologic examination revealed a metastatic RCC which was a poorly differentiated clear cell variant lesion, being consistent with a metastasis of RCC resected 11 years ago. Chest X-ray showed two metastases with a maximum size of 1 and 3.5 cm respectively (Figure 1). Computed tomography (CT) scans of the abdomen displayed a gastric mass (5 cm in diameter), several para-aortic lymphadenopathies and liver metastases (Figure 2).

Chemotherapy was proposed to reduce the tumor size as well as the hepatic and pulmonary metastases, but the patient refused to undergo any treatment and was

Table 1 Characteristics of all reported patients with RCC who developed isolated metastatic gastric lesions

Patients (n)	Appearance after nephrectomy	Outcome
1	7-years	3-year DFS
1	7-years	Not available
1	4-years	17-mo DFS
1	6-years	> 5-mo survival
6	Not known	Autopsy study
2	Not known	Autopsy study
1	9-years	Not available
1	Not known	Autopsy study
1	At nephrectomy	3-mo survival
1	13-years	2-year survival

RCC: Renal cell carcinoma; DFS: Disease-free survival.

discharged.

After one month, he presented again to the emergency room with postural dizziness, melena, and weakness. The patient eventually underwent surgery. The large mass described above was found in the posterior wall of the fundus and body of the stomach. It was penetrating the surrounding tissues. Many lymphadenopathies along the common hepatic artery, left gastric artery and celiac trunk were also present. Therefore, total gastrectomy was performed with lymphadenectomy and omentectomy, and esophago-jejunostomy according to the Roux technique. Histopathological examination confirmed the presence of metastatic clear RCC. Both proximal and distal resection margins appeared to be tumor free. The patient had an uneventful postoperative course.

One month after the intervention, he was started on chemotherapy with gemcitabine and IL-2. The therapy lasted for 20 mo. The patient during this period had a good quality of life. Because of the presence of abdominal pain, a treatment with morphine was started. He also received erythropoietin and iron to treat anemia. Then he received blood transfusions in the following 3 mo. The patient finally died due to the worsening of anemia and cachexia.

DISCUSSION

This paper describes for the first time an uncommon biological and clinical behavior of RCC. RCC is known to cause metastatic recurrences many years after its resection and even after immunotherapy^[14]. However, while recurrence at the pancreatic site is quite common, gastric recurrence is atypical^[15]. Metastatic involvement of the stomach is usually considered as an extraordinary event and accounts for only 0.2%-0.7% of gastric neoplastic diseases. In a large series consisting of 23 019 autopsies, Davis and Zollinger^[16] found 67 metastases to the stomach from primary tumors outside the gastrointestinal tract. None was from the kidney. Higgins^[17] reported 64 metastases in the stomach out of 31 541 examined autopsies. In this series, none was from the kidney.

An isolated gastric metastasis from RCC is extremely rare and only 15 cases including the present one have been described^[6-14]. The characteristics of all the reported cases

including our one are summarized in Table 1. From Table 1, we could see that RCC metastasizes to the stomach several years after nephrectomy.

Gastric RCC metastasis often starts as a submucosal lesion, which encroaches onto the mucosa and becomes ulcerated. The lesion may be single or multiple and is grossly polypoid or plaque-like. Metastatic tumors can be distinguished from gastric carcinoma based on the absence of cellular atypia in the gastric glandular structures which may appear compressed by the metastatic tumor. The most common presenting symptom is abdominal pain but nausea, vomiting, and gastrointestinal bleeding can also be present. Gastrointestinal bleeding is mainly related to acid erosion of the metastatic lesion. Diagnosis is confirmed by upper gastrointestinal endoscopy and histopathological examination of a biopsy sample of the lesion.

Surgical excision of gastric metastasis is mandatory as these lesions may bleed again after endoscopic coagulation treatment. A unique metastasis should be treated as a new tumor with prompt surgical excision. This often results in a significant survival prolongation with a good quality of life. In the present case, though multiple pulmonary and hepatic metastases were present, surgery had to be performed because of the high risk of rebleeding.

Compared to previously reported cases, our patient had the longest disease-free interval from the resection of RCC to the stomach metastasis as he developed recurrence at the pancreatic site 10 years after the resection of RCC and gastric metastasis 1 year later.

The prolonged disease-free time could be at least in part due to the IL-2 immunotherapy that apparently yielded a complete response. Immunotherapy with IL-2 is in fact considered as the mainstay of treatment for RCC as cytotoxic chemotherapy fails^[18]. This treatment can achieve certain effects in patients with a metastatic disease^[19]. The mechanism of action of IL-2 at the cellular level is not perfectly known. It is hypothesized that IL-2 stimulates a cellular immune response to tumor involving mainly the natural killer cells and T lymphocytes^[20]. The response rate fluctuates from 15% to 20% and complete responders account for one-third of this group^[19]. The survival time of complete responders is significantly prolonged and exceeds 60% at 5 years, while non-responders with metastatic RCC have a median survival time of less than 1 year^[19].

The present case draws attention to an important aspect of the biological behavior of RCC. This tumor is often a slow-growing lesion and may be associated with a late onset of solitary metastases that may occur even 11 years later than the primary tumor. Usually metastases grow slowly. In our patient, the first pancreatic recurrence was 1 year earlier than the gastric one. Gastric localization was undetectable when the patient underwent surgery for the first recurrence. Therefore, a careful follow-up should be made in patients who develop

a first recurrence of RCC since they may develop multiple recurrences at different sites even after several years. IL-2 therapy seems to be able to treat the disease once it spreads out but has no effect on its prevention.

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Pharmacokinetics of paclitaxel in a hemodialysis patient with advanced gastric cancer: A case report

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Abstract

We report for the first time the possibility of weekly paclitaxel chemotherapy for a patient with advanced, nonresectable gastric cancer undergoing hemodialysis. A 50-year-old man with chronic renal failure due to bilateral polycystic kidneys, who had undergone hemodialysis three times a week for 5 years, presented with hematemesis in December 2004. Based on the diagnosis of gastric cancer with lymph node metastases, surgery was performed. On the 15th postoperative day, the patient was treated with chemotherapy using paclitaxel. Paclitaxel was administered at a dose of 60 mg/m² as a 1 h iv infusion in 250 mL of saline. Hemodialysis was started 1 h after the completion of the paclitaxel infusion and was performed for 3 h. Paclitaxel was administered weekly on d 1, 8, and 15 on a 28-d cycle. The maximum plasma concentration of paclitaxel was 1390 µg/L. The area under the curve of paclitaxel was 4398.6 µg h/L. Grade 2 leukopenia was encountered during the first cycle. The plasma concentrations of paclitaxel from 6 to over 24 h after the infusion were 0.01 to 0.1 µmol/L in our patient, and these concentrations have been shown to be effective on inhibiting the growth of gastric cancer cells without producing adverse side effects in the patient. The plasma concentration of paclitaxel was not influenced by hemodialysis. We conclude that the pharmacokinetics of paclitaxel is not altered in a patient with renal failure, and that weekly paclitaxel is a suitable treatment regimen for hemodialysis patients with advanced gastric cancer.

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Key words: Paclitaxel; Gastric cancer; Hemodialysis

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INTRODUCTION

The necessity of chemotherapy for hemodialysis patients with malignancies has increased as the life span of hemodialysis patients has grown longer because of improvements in hemodialysis. The median survival time of patients receiving the best supportive care for advanced, nonresectable gastric cancer (AGC) is only 3 to 4 mo. Some randomized studies have demonstrated the superiority of chemotherapy over best supportive care for improving patient prognosis, and the significance of performing chemotherapy for patients with AGC is now recognized^[1,2]. Although TS-1 has frequently been used for patients with AGC as a first line chemotherapy in Japan, this drug is prohibited for use in patients with renal failure because adverse reactions, such as bone marrow depression, may be enhanced. In this case report, we describe for the first time the possibility of weekly paclitaxel chemotherapy for a hemodialysis patient with AGC.

CASE REPORT

A 50-year-old man with chronic renal failure due to bilateral polycystic kidneys, who had undergone hemodialysis 3 times a wk for 5 years, presented with hematemesis in December 2004. Endoscopic examination demonstrated a large tumor on the lesser curvature of the antrum. Biopsy specimens of the tumor revealed poorly differentiated adenocarcinoma. Computed tomography of the abdomen showed enlarged lymph nodes surrounding the stomach and displayed bilateral polycystic kidneys. Based on the diagnosis of gastric cancer with lymph node metastases, surgery was performed. On entering the peritoneal cavity, peritoneal disseminations were encountered. Although a curative intervention was not possible, distal gastrectomy was performed to prevent bleeding from the cancerous lesion. On the 15th postoperative d, the patient was treated with chemotherapy using paclitaxel.

Paclitaxel was administered at a dose of 60 mg/m² as a 1 h iv infusion in 250 mL of saline. The following premedication was administered as a 60 min pretreatment prior to paclitaxel: dexamethasone, 10 mg iv; chlorphenamine, 10 mg iv; and ranitidine, 50 mg iv. Hemodialysis was started 1 h after the completion of the paclitaxel infusion and was performed for 3 h. Paclitaxel was administered weekly on

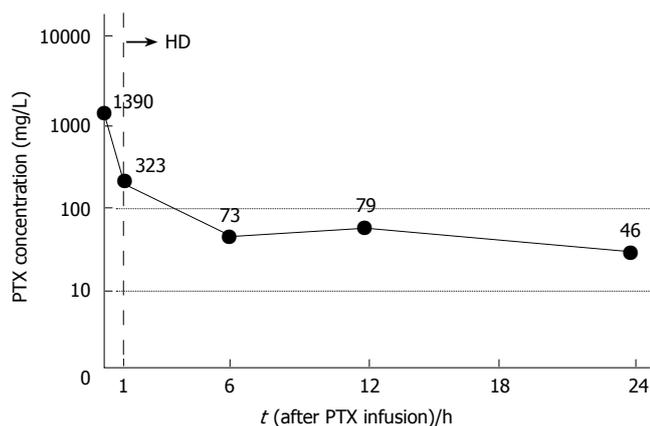


Figure 1 The plasma concentrations of paclitaxel following iv infusion. PTX: Paclitaxel; HD: Hemodialysis.

d 1, 8, and 15 on a 28-d cycle.

The plasma concentrations of paclitaxel following iv infusion are shown in Figure 1. The maximum plasma concentration (C_{max}) of paclitaxel was 1390 $\mu\text{g/L}$. The area under the curve (AUC) of paclitaxel was 4398.6 $\mu\text{g h/L}$. Grade 2 leukopenia occurred during the first cycle. Chemotherapy had to be stopped because bleeding from the jejunum near the anastomosis occurred, in spite of the fact that the platelet count was normal during the first cycle. Unfortunately, peritonitis carcinomatosa occurred, and the patient died in April 2005.

DISCUSSION

Anticancer drugs such as 5-FU, cisplatin, and irinotecan hydrochloride are effective for gastric cancer, but in a patient with hemodialysis-dependent renal failure, the dosages of these drugs must be carefully chosen because of potential side effects arising from the increased blood concentration of these drugs. Paclitaxel is the first drug from a group of drugs that inhibit microtubule disassembly and is extensively metabolized by the liver and secreted in bile, with less than 10% extracted by the kidneys^[3,4]. As a single agent, phase II study results on gastric cancer have demonstrated an overall response rate of approximately 15% to 20%^[5,6]. Paclitaxel has a cytotoxic effect on human gastric cancer cell lines in a dose- and time-dependent manner. Chang *et al*^[7] reported that exposure of gastric cancer cells to 0.01 $\mu\text{mol/L}$ of paclitaxel for 24 h appeared to be cytotoxic. On the other hand, some investigators have reported that a paclitaxel threshold of 0.1 $\mu\text{mol/L}$ was informative with respect to neutropenia, and that the total dose and AUC did not correspond with the incidence or severity of neutropenia^[8,9]. In our phase I study of weekly paclitaxel and doxifluridine in AGC patients, the recommended dose of paclitaxel was 80 mg/m^2 plus doxifluridine at 533 mg/m^2 . However, the effective rate was 33.3% with no adverse events at a paclitaxel dose of 60 mg/m^2 ^[10,11]. Therefore, we decided to use a dose of 60 mg/m^2 of paclitaxel for the first cycle to ensure the safety of our patient. To our knowledge, there are a small number of reports in the

literature on the treatment of advanced ovarian cancer with paclitaxel in patients with renal failure, but there are no reports of chemotherapy with paclitaxel in an AGC patient with renal failure^[12,13]. The plasma concentrations of paclitaxel at 6 to over 24 h after the infusion were 0.01 to 0.1 $\mu\text{mol/L}$ in our patient, and these concentrations have been shown to be effective on inhibiting the growth of gastric cancer cells without producing adverse effects for patients. Kim *et al*^[14] reported the pharmacokinetic analysis in the treatment of relapsed breast cancer by weekly paclitaxel. In that report, the peak concentrations at 0 min (C_{max}), 30 min, and 24 h in patients treated with a dose of 60 mg/m^2 of paclitaxel were 2.18 ± 0.68 , 0.65 ± 0.20 , and 0.017 ± 0.012 $\mu\text{mol/L}$, respectively. These findings are similar to our results and suggest that the plasma concentration of paclitaxel is not influenced by hemodialysis.

Chemotherapy has to be stopped because of bleeding from the postoperative peptic ulcer. Neither thrombocytopenia nor coagulopathy occurs as a result of chemotherapy. We conclude that the pharmacokinetics of paclitaxel is not altered in a patient with renal failure and that weekly paclitaxel is a suitable treatment regimen for patients with AGC on hemodialysis.

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CASE REPORT

Development of ulcerative colitis during the course of rheumatoid arthritis: Association with selective IgA deficiency

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INTRODUCTION

Recent case reports described the development of ulcerative colitis (UC) in patients with rheumatoid arthritis (RA)^[1,2]. Although UC is commonly associated with arthritic manifestations such as ankylosing spondylitis and sacroiliitis^[3], RA complicated by UC is uncommon^[4], hence the underlying mechanisms of the association between UC and RA remain unclear. Differentiation between UC and colitis, primarily or secondarily related to RA or drug-induced colitis associated with medications for RA, can be difficult sometimes^[5].

Selective deficiency of immunoglobulin A (IgA) is the most frequent hypogammaglobulinemia^[6]. It is associated with increased risk of autoimmune diseases including RA^[6,7]. One might speculate that IgA deficiency plays a role in the mucosal immune and inflammatory responses of UC^[6]. Herein, we describe the development of UC in a patient with longstanding RA and selective IgA deficiency. To the best of our knowledge, this is the first case report on the association of RA with UC and IgA deficiency.

Abstract

A 56-year-old woman with a 29-year history of rheumatoid arthritis (RA) was admitted to the hospital, complaining of high fever, abdominal pain and severe bloody diarrhea. Colonoscopy revealed friable and edematous mucosa with spontaneous bleeding, diffuse erosions and ulcers extending from the rectum to the distal transverse colon. Histopathological findings of rectal biopsies were compatible with ulcerative colitis (UC). Being diagnosed as having severe active left-side UC, she was successfully treated with intravenous methylprednisolone followed by prednisolone and leukocytapheresis. Laboratory tests revealed low serum and saliva IgA levels, which might play a role in the development of UC. To our knowledge, this is the first case of UC occurring during the course of RA, accompanied by selective IgA deficiency.

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Key words: Ulcerative colitis; Rheumatoid arthritis; Selective IgA deficiency

Asada Y, Isomoto H, Shikuwa S, Wen CY, Fukuda E, Miyazato M, Okamoto K, Nakamura T, Nishiyama H, Mizuta Y, Migita K, Ito M, Kohno S. Development of ulcerative colitis during the course of rheumatoid arthritis: Association with selective IgA deficiency. *World J Gastroenterol* 2006; 12(32): 5240-5243

CASE REPORT

A 56-year-old woman who had been diagnosed with UC at a local clinic 5 mo earlier, was admitted to our hospital, complaining of high fever, exacerbated lower abdominal pain and a 4-wk duration of bloody diarrhea, despite treatment with 30 mg/d of oral prednisolone. She had suffered from RA for 29 years and the current stage and class of RA, based on Steinbrocker's classification, was IV and II, respectively. RA had been stable for the last 5 years under continuous treatment consisting of bucillamine (100 mg/d), prednisolone (5 mg/d) and D-penicillamine (600 mg/d). She had a history of adverse events of proteinuria and skin eruption with itching against bucillamine and mesalazine, respectively. She had not taken any other medications such as antibiotics, non-steroid anti-inflammatory drugs (NSAIDs) or gold salts, prior to the development of UC. She was a non-smoker, did not drink alcohol and had not undergone previous gastrointestinal surgery.

On admission, physical examination revealed body weight of 32 kg, height of 146 cm, body temperature of 37.9°C, regular pulse rate of 96/min and blood pressure of 148/76 mmHg. Conjunctiva palpebra and bulbus were

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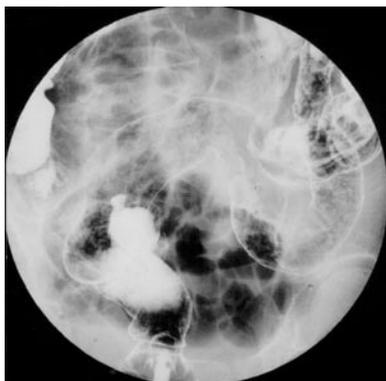


Figure 1 Barium enema shows loss of mucosal pattern with speculation, absent haustration, narrowing and shortening of the bowel, extending from the rectum to the distal transverse colon.

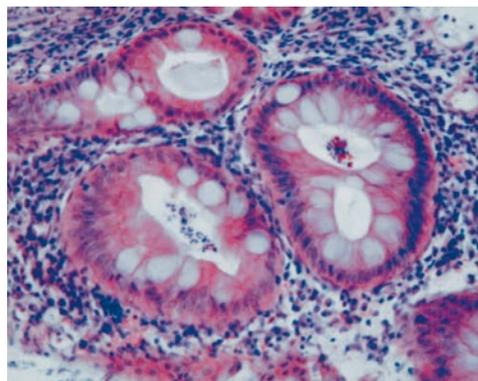


Figure 3 Histopathological examination of biopsied rectal mucosa showed severe neutrophil infiltration, goblet cell depletion and mild crypt abscess (hematoxylin and eosin staining).

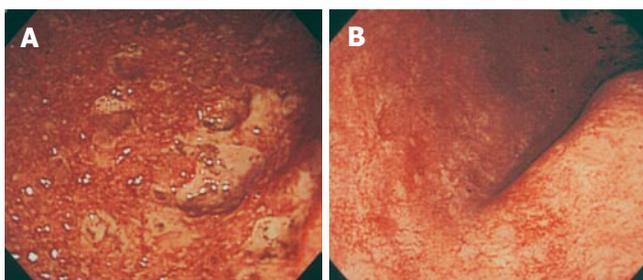


Figure 2 (A) Sigmoidoscopy revealed friable, edematous mucosa with granularity, mucous exudates and bleeding throughout the rectum and sigmoid colon. Multiple erosions and punctate ulcers were observed without normal-appearing intervening mucosa. (B) Repeated colonoscopy before discharge revealed almost normal mucosa from the distal transverse colon to the rectum.

normal without pallor or jaundice. Heart sounds were clear with regular sinus rhythm and no rales were audible in the lung fields. The abdomen was tender on palpation in the lower quadrant with weak bowel sounds, but neither muscular guarding nor Blumberg's sign was noted. Digital rectal examination revealed dark red blood. There was no edema on the face or extremities and there were no signs of motor or sensory disturbances. Typical joint deformities and morning stiffness of RA were noted in both hands.

Laboratory investigations showed leukocyte count, $10\,900/\text{mm}^3$ (normal, $3500\text{--}9100$); platelet, $30.6 \times 10^4/\text{mm}^3$ (normal, $13.0\text{--}36.9 \times 10^4$); hemoglobin (Hb), 132 g/L (normal, 113–152); C-reactive protein (CRP), 6.6 g/L (normal, < 0.3); serum total protein, 53 g/L (normal, 65–82); albumin, 23 g/L (normal, 37–52); IgG, 16.58 g/L (normal, 8.7–17); IgA, 0.2 g/L (normal, 1.1–4.1); IgM, 0.63 g/L (normal, 0.46–2.6); rheumatoid factor, 48 000 IU/L (normal, 0–20 000); passive agglutination (RAPA), 1:80 (normal, $< 1:40$); antinuclear antibodies < 40 (normal, < 40); CH50, 23.2 g/L, (normal, 25–48); and C3, 0.63 g/L (normal, 0.86–1.6). Saliva IgA level was low at 37.6 mg/L (normal, 51.6–931). Urinalysis was normal and no infectious agents were identified in stool specimens. *Clostridium difficile* toxin was negative in feces.

Chest X-ray revealed no abnormalities such as pneumonia and interstitial pneumonitis. Abdominal plain X-ray revealed a small amount of bowel gas without formation of air-fluid level or free air and no toxic dilation of the colon was seen. Hand X-rays revealed bilateral involvement of most metacarpal phalangeal joints with destruction and deviation, whereas systemic skeletal X-ray

study showed no abnormalities in cervical and lumbar spine and hip joints. Barium enema showed disappearance of haustra coli, narrowing and shortening of the affected colon, and uneven surface with spiculation, extending from the rectum to the distal transverse colon (Figure 1). The terminal ileum and the ascending and proximal transverse colon were intact. Barium study of the small intestine and upper gastrointestinal endoscopy were normal. Contrast computed tomography of the abdomen and pelvis showed diffuse mural thickening of the affected colon. Initial sigmoidoscopy performed on the next hospitalization day revealed friable, edematous mucosa with granularity, mucous exudates and spontaneous bleeding throughout the rectum and sigmoid colon. Diffuse erosions and punctate ulcers were noted with lack of normal-appearing intervening mucosa (Figure 2A). Histopathology of colorectal biopsies showed diffuse mucosal infiltration of inflammatory cells, deformed atrophic crypts, goblet cell depletion and crypt abscess (Figure 3). No granulomas, inclusion bodies, vasculitic lesions or amyloid deposits were detected under microscopic examination of hematoxylin-eosin and Congo-red stained sections (data not shown). Based on the clinical, radiological, endoscopic, and histopathological findings, the patient was diagnosed as having severe active, left-sided UC.

Under total parenteral nutrition, she received a course of intravenous methylprednisolone pulse therapy (1 g/d for 3 consecutive days), followed by large doses of intravenous prednisolone, starting with 50 mg/d. Since cytomegalovirus antigenemia was detected 7 d after admission, she also received a 14-d course of intravenous gancyclovir injection at 400 mg/d.

She also received leukocytapheresis (LCAP) therapy, employing a commercially available leukocyte removal column (Cellsorba, Asahi Medical, Tokyo) once a week for 10 successive weeks. Such intensive treatment led to dramatic improvement of clinical symptoms and signs without any adverse reaction and to normalization of laboratory tests. Follow-up colonoscopy 3 wk later revealed marked improvement, and normal colonic mucosa at discharge from the hospital three months after admission (Figure 2B). The RA disease status was unchanged during hospitalization and thereafter. At the last visit to the outpatient clinic, 6 mo after discharge, the patient was still

in complete remission of colitis under 10 mg/d of oral prednisolone.

DISCUSSION

When colitis appears in RA patients, drug-induced colitis, ischemic colitis due to vasculitis associated with RA and secondary amyloidosis must be ruled out first^[8-12]. It is known that therapeutic agents for RA such as NSAIDs, gold salts and D-penicillamine give rise to various types of gastrointestinal complications, evidences indicate that colitic lesions are associated with these anti-RA drugs^[8-10]. Although Langer *et al*^[9] reported a case of gold salts-induced colitis resembling UC, our patient was not treated with gold salts or NSAIDs. There are also reports on D-penicillamine-induced colitis^[10], with rapid recovery after cessation of the agent. Although the patient had used penicillamine for 6 years, colitis had never appeared. Moreover, her colitis improved despite continuous use of this drug. Thus, anti-RA medications are unlikely to be the underlying cause of colitis in our patient.

Rheumatoid vasculitis (also known as malignant RA) affects a variety of organs including kidneys, lungs and gastrointestinal tract^[13]. Previous studies indicate that clinically apparent rheumatoid vasculitis occurs in less than 1% of RA patients and intestinal involvement is noted in about 20% of such cases^[11]. The gastrointestinal lesions associated with rheumatoid vasculitis are characterized by multiple sharply-defined ulcers, typically spared by normal-appearing mucosa, with a predilection for the small intestine, the sigmoid colon and cecum^[8,11]. Burt *et al*^[11] described a patient with RA who had colonic vasculitis presenting as pancolitis similar to UC. In that patient, however, repeated colonoscopy following treatment with prednisolone showed that the intervening mucosa among superficial ulcers was normal, allowing differentiation of colitis due to RA-related vasculitis from idiopathic UC. Histopathological examination of multiple biopsies taken from colorectal mucosa identified no vasculitic lesions in our patient, who showed no other vasculitic diseases such as scleritis and interstitial pneumonitis^[13,14].

Recent clinical studies have indicated that the incidence of amyloidosis confirmed by biopsies in RA patients is about 10%^[12]. Kato *et al*^[15] reported a suggestive case of gastrointestinal amyloidosis secondary to RA manifested as pancolitis resembling UC. In our patient, however, rectal biopsy specimens showed diffuse infiltration of mono- and poly-nuclear leukocytes and crypt abscess, which were compatible with UC, but no intramucosal amyloid deposits. In addition, neither microbiological nor histopathological examinations showed any pathognomonic findings in this case, thus allowing differentiation between UC and other possible inflammatory bowel conditions.

Being diagnosed as having severe active UC extending to the left-side colon, the patient was treated with intravenous methylprednisolone and subsequently with prednisolone. Given that the patient had already received oral prednisolone even at 30 mg/d with exacerbation of UC, she was also treated with LCAP. In previous studies, LCAP frequently led to significant improvement of UC that had been intractable to steroids^[16,17]. A recent open-

label multicenter study demonstrated that LCAP can be more effective than high-dose steroid treatment with fewer adverse effects^[17]. Currently, LCAP is used as a treatment modality for active UC. Although histopathological examination of colorectal biopsies showed no inclusion bodies, cytomegalovirus antigenemia was detected, allowing our patient to undergo additional treatment with intravenous gancyclovir. We believe that the intensive combination therapies led to complete clinical and endoscopic remission of severe and steroid-refractory colitis.

Arthritic manifestations represent the most common extraintestinal manifestations of inflammatory bowel diseases, affecting 31% and 36% of patients with UC and Crohn's disease, respectively^[5]. Coexistence of RA and UC is, however, rare and no more than 0.14% to 0.8% of UC patients suffer from RA^[4,18]. Larger population-based studies are warranted to unravel the mechanisms underlying the association between UC and RA. In this regard, accumulation of HLA data may provide a potential clue for this relationship^[19].

In the present case, serum concentrations of IgA, but not IgG or IgM, were constantly low ranging from 1 to 20 mg/dL and reduction of secretory IgA in saliva was also noted during hospitalization. Systemic and local reduction of IgA might result in weakening of the mucous barrier against intestinal flora and enhance exposure of the mucosal immune system to intestinal bacteria or the toxic components, eventually leading to sustained inflammation within the colorectal mucosa^[4]. However, the precritical serum IgA level had not been estimated in our patient, and the etiology of selective IgA deficiency remains elusive as to whether it occurred primarily or not. In fact, secondary IgA deficiency may be caused by such anti-RA drugs as D-penicillamine, steroids, both of which had been prescribed for her RA, sulfasalazine and gold salts^[7,20].

Liblau *et al*^[6] reported that the frequency of autoimmune diseases in IgA deficient patients ranged from 7 to 36%, supporting the notion that selective IgA deficiency may be a risk factor of organ-specific and systemic autoimmune diseases. A recent study showed that the prevalence of IgA deficiency in RA patients was quite low (0.3%)^[6], whereas Badcock *et al*^[7] reported that IgA deficient patients were more likely to have a history of RA in the first-degree relatives, suggesting inheritance of predisposing factor(s). Of note, Snook *et al*^[4] reported that 6.6% of UC patients had at least one autoimmune disorder and the prevalence of a specified group of autoimmune disorders was three times greater than expected in patients with UC.

In conclusion, we described the first case of RA, complicated with UC and selective IgA deficiency. Underlying immune dysfunction, possibly in addition to genetic predisposition, might play a role in these associations.

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CASE REPORT

Prolonged cholestasis after raloxifene and fenofibrate interaction: A case report

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Abstract

Assigning causality in drug-induced liver injury is challenging particularly when more than one drug could be responsible. We report a woman on long-term therapy with raloxifene who developed acute cholestasis shortly after starting fenofibrate. The picture evolved into chronic cholestasis. We hypothesized that an interaction at the metabolic level could have triggered the presentation of hepatotoxicity after a very short time of exposure to fenofibrate in this patient. The findings of an overexpression of vascular endothelial growth factor in the liver biopsy suggest that angiogenesis might play a role in the persistence of toxic cholestasis.

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Key words: Raloxifene; Fenofibrate; Drug-drug interactions; Hepatotoxicity; Causality assessment

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INTRODUCTION

Fibrates are extensively prescribed for patients with pri-

mary hypertriglyceridemia. Raloxifene, a selective estrogen receptor modulator that prevents bone resorption without causing endometrial or breast cancer, is effective in the treatment of osteoporosis in postmenopausal women. Both agents are widely used in clinical practice, thus posing an increased likelihood of their concomitant prescription. Although hepatotoxicity is a rare phenomenon associated with their use^[1-3], we report a patient on long term therapy with raloxifene who developed prolonged cholestatic hepatitis shortly after the introduction of fenofibrate.

CASE REPORT

A 60-year-old woman was started on raloxifene hydrochloride (60 mg/d) in 2000 for the prevention of osteoporosis. On December 1st 2003 she was prescribed fenofibrate (250 mg/d) for hypertriglyceridemia (triglyceride level 423 mg/dL). Her liver function was normal and she had no toxic habits, no drug allergies and was not taking other drugs. There was no family history of cholestatic and non-cholestatic diseases of the liver and biliary tract. She did not suffer from diabetes mellitus or pancreatitis and was moderately obese (BMI 26.7). On December 14th she noticed dark urine and both drugs were discontinued, no attempt of drug reintroduction was recorded. On admission (December 16), she was afebrile and jaundiced. Aspartate aminotransferase (AST) was 153 U/L (normal < 30), alanine aminotransferase (ALT) 241 U/L (normal < 36), alkaline phosphatase (AP) 174 U/L (normal < 104), gamma-glutamyltransferase (GGT) 271 U/L (normal < 32), and total bilirubin 11.07 mg/dL with direct bilirubin 9.6 mg/dL. The leucocyte count was $3.3 \times 10^9/L$ with 17.7% lymphocytes. Serology ruled out viral causes and screening for autoantibodies was negative. Imaging testing including a magnetic resonance cholangiography showed cholelithiasis with no other pathological findings (i.e. gallstones, tumour). A liver biopsy showed a moderate inflammatory infiltrate of lymphocytes with hepatocellular cholestasis and focal necrosis. Immunohistochemistry showed that expression of vascular endothelial growth factor (VEGF) (clone C-1, Santa Cruz Biotechnology, IC, USA) resulted in mild to moderate granular staining in hepatocytes of zone 3 (Figure 1A). Immunohistochemical staining was performed as previously described^[4]. Ten days later a morbiliform and very pruriginous rash appeared in the lower extremities that progressively generalized to the trunk, upper extremities and face.

Liver enzymes decreased initially but on January 20, 2004, the total bilirubin peaked at 21.84 mg/dL (direct

Table 1 Liver tests while on raloxifene therapy and follow-up after raloxifene and fenofibrate interaction during 14 d of coadministration

Admission date	Nov 10, 2003	Dec 16, 2003	Dec 22, 2003	Jan 2, 2004	Jan 20, 2004	Mar 1, 2004	April 16, 2004	July 2, 2004	Aug 13, 2004	February, 2005	August, 2005
Serum Bilirubin (mg/dL)											
Total	-	11.07	14.91	14.33	21.84	8.31	2.81	0.72	0.78	0.65	0.83
Direct	-	9.58	14.07	13.69	19.72	7.72	1.79	0.14	-	-	-
AST (U/L)	26	153	88	36	321	97	106	84	84	45	53
ALT (U/L)	16	241	167	50	382	160	162	91	74	45	60
GGT (U/L)	21	271	239	97	1246	1387	539	1279	1255	751	666
Alkaline phosphatase (U/L)	72	174	236	240	800	420	490	1195	793	493	444
Lymphocytes (%)	26	17.7	13.8	11.5	5.9	14.3	17.7	-	27.7	33.9	32.20
VEGF (pg/mL)	-	-	-	-	144	-	-	244	-	-	-

Treatment was stopped on 14 December 2003. Total bilirubin ($n < 1.8$ mg/dL); AST: Aspartate aminotransferase ($n < 30$ U/L); ALT: Alanine aminotransferase ($n < 36$ U/L); GGT: Gamma-glutamyl transpeptidase ($n < 32$ U/L); Alkaline phosphatase ($n < 104$ U/L); lymphocytes ($n = 20\%-40\%$); VEGF: Plasma vascular endothelial growth factor.

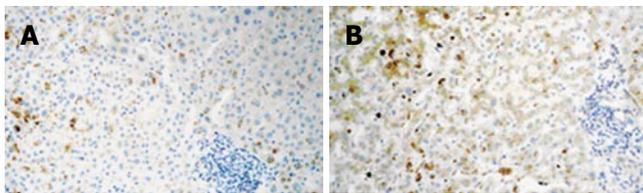


Figure 1 A: The first liver biopsy in December 2003 showing mild to moderate granular stain in hepatocytes of zone 3; B: The second liver biopsy in February 2004 showing diffuse VEGF stain with predominance of zone 3 (original magnification 200 x).

bilirubin 19.7 mg/dL), with increases in ALT, GGT and AP values (Table 1). Lymphocytes were 0.077×10^9 /L (5.9%). The multifomed erythema worsened at this time with subsequent resolution. In February, a second liver biopsy was performed (Figure 1B). The liver specimen showed intense hepatocellular and canalicular cholestasis, mainly in zone 3, with mild to moderate lymphocytic infiltrate in portal tracts (one of them with focal interface hepatitis). Biliary ducts were absent in three out of the 7 portal tracts present in the biopsy. In this biopsy VEGF showed diffuse positivity which was more intense in zone 3, and Kupffer cells were also reactive. At this time, EDTA plasma VEGF levels (determined using a commercially available ELISA kit, R&D Systems, MN, USA) were 144 pg/mL (healthy subjects levels < 80 pg/mL)^[5]. On July 1, 2004, ALT was 91 U/L, AP 1195 U/L, GGT 1279 U/L, and total bilirubin 0.72 mg/dL. Plasma VEGF values increased to 244 pg/mL. ALT, GGT and AP levels still remained high 21 mo after the initial episode (Table 1).

DISCUSSION

Assigning a causal relationship to a drug associated with hepatic injury remains a major challenge, especially when it is the first report of a particular reaction and when more than one drug could be the culprit. If treatments are not started simultaneously, common sense and causality assessment methods tend to incriminate the last drug introduced, as the fenofibrate in our patient. Actually,

fenofibrate scored higher than raloxifene when the CIOMS scale was applied to yield 9 and 6 points which fell in the category of highly probable and probable, respectively.

However, this issue may not always be so straightforward and other considerations should be born in mind. Acute hepatitis is rarely related to fenofibrate and the reported cases do not reflect the type of injury that is presented here. Most cases present with hypergammaglobulinemia and high titers of anti-nuclear antibodies, and on liver biopsies a lympho-plasmocytic infiltrate, resembling type I auto-immune hepatitis, is evident^[1]. The chronic forms of liver damage are more exceptional, usually appearing after long periods of exposure. They show different histopathological findings such as chronic active hepatitis with bridging necrosis or a reduction in the number of interlobular bile ducts in a clinically asymptomatic patient^[2]. On the contrary, the only published case of raloxifene-associated hepatitis did exhibit a late peak of bilirubin one month after drug withdrawal^[3], similarly to our patient.

To our understanding, this patient suffered from hepatic toxicity due to an interaction between raloxifene and fenofibrate that could result in liver toxicity by altering the threshold for exposure to toxic metabolites. Both compounds are highly protein bound to albumin with the potential of competitive drug displacement^[7], and an irreversible inhibition of CYP3A4 by raloxifene has been described which is more frequently associated with unfavorable drug-drug interactions^[8,9].

The prolonged course of the abnormalities in liver biochemistry deserves further consideration. Indeed, an immune mechanism is suggested in the patient by the presence of a severe toxic cutaneous reaction and cytopenia. In these circumstances, a self propagating immune response may persist, which might explain the outcome^[6].

An interesting finding in this case was the over-expression of VEGF (the most potent proangiogenic growth factor) in the liver and increased plasma VEGF levels, when the clinical and biological picture was in remission and the hepatic lesion evolved into a chronic

cholestatic phase. This suggests that angiogenesis may be an important mechanism involved in the persistence of toxic cholestasis which is up-regulated in response to tissue damage and release of pro-inflammatory cytokines. This has been recently shown in primary biliary cirrhosis^[10]. Indeed, angiogenesis is a novel mechanism involved in chronic liver damage and its role in drug-induced liver injury also deserves to be defined.

In summary, an interaction between raloxifene and fenofibrate may occur in a postmenopausal woman with resulting hepatotoxicity. Clinicians should be aware of this adverse reaction and patients should be followed up closely. Clinical judgment of the attribution of causality must be made, especially in particularly troublesome cases in which major drug metabolism mechanisms and the potential for pharmacokinetic drug interactions should always be kept in mind.

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Spinal cord compression secondary to bone metastases from hepatocellular carcinoma

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Abstract

Bone metastases are rare in primary hepatocellular carcinoma (HCC). Spinal cord compression (SCC) due to bone metastases occur commonly in patients with lung and breast carcinomas, and metastatic HCC is an unusual cause of SCC. Spinal cord compression is an oncologic emergency and treatment delays can lead to irreversible consequences. Thus, the awareness that SCC could be a potential complication of bone metastases due to HCC is of significance in initiation of early treatment that can improve the quality of life and survival of the patients, if diagnosed earlier. This paper describes four cases of primary HCC with varied manifestations of SCC due to bone metastases. The first patient presented primarily with the symptoms of bone pains corresponding to the bone metastases sites rather than symptoms of associated hepatic pathology and eventually developed SCC. The second patient, diagnosed as having HCC, developed extradural SCC leading to paraplegia during the course of illness, for which he underwent emergency laminectomy with posterior fixation. The third patient developed SCC soon after the primary diagnosis and had to undergo emergency laminectomy. Post laminectomy he had good neurological recovery. The Fourth patient presented primarily with radicular pains rather than frank paraplegia as the first manifestation of SCC.

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Key words: Alpha fetoprotein; External beam radiotherapy; Hepatocellular carcinoma; Laminectomy; Spinal cord compression

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary tumor of the liver and has a mean survival of less than one year after primary diagnosis, if left untreated^[1]. Extra-hepatic spread of HCC occurs commonly to the lungs (37%-70%) and regional lymph nodes (23%-45%)^[1]. The reported incidence of bone metastases is comparatively low (2%-20%)^[2-5]. However, an incidence of 28% of bone metastases from HCC has been reported by Katyal *et al*^[1]. The most frequent sites of bone metastases reported by Kuhlman *et al* were ribs, spine, femur, pelvis and humerus^[2]. Spinal cord compression (SCC) is a rare event in the natural course of metastatic HCC as evidenced by Bharat Kantharia *et al* in their study on HCC^[6]. Review of literature shows only a few patients with HCC who developed radiculopathy or SCC secondary to bone metastases. SCC is an oncologic emergency and awareness that SCC could be a potential complication of bone metastases, can improve the survival and quality of life of these patients, if treated promptly. We herein report four cases of pathologically confirmed primary HCC with spinal metastases who developed extradural SCC.

CASE REPORTS

Case 1

A 55-year-old normotensive, nondiabetic, non-alcoholic man with no history of chronic liver disease, presented with the complaints of low backache and generalized weakness for 20 d. Clinically, he had tenderness in the presacral region. Further investigations revealed normal hematology and biochemical parameters. Magnetic resonance imaging (MRI) of the whole spine showed a tumor deposit in the presacral area. Whole body 99mTc bone scan revealed increased radiotracer uptake in right ilium, sacrum including both sacroiliac joints and D3 vertebra. Computerised Tomography (CT) guided biopsy and fine needle aspiration cytology (FNAC) from the presacral tumor were consistent with the metastatic

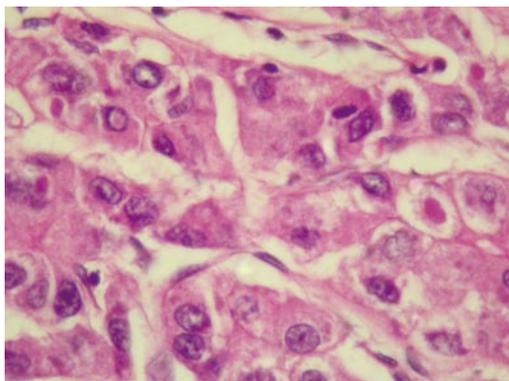


Figure 1 Tru cut biopsy of liver lesion showing increased trabecular thickness, cellular pleomorphism with vesicular nuclei. Sinusoids are lined by flat endothelial cells, Kupffer cells are absent.

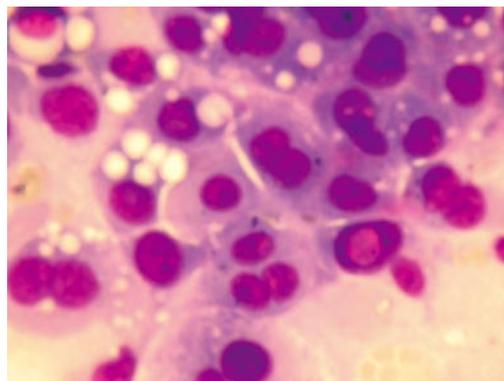


Figure 3 FNAC of vertebral metastasis showing cellular monomorphism, moderate pleomorphism with occasional intranuclear inclusion.

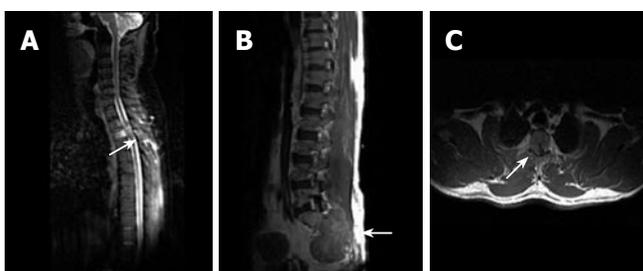


Figure 2 **A:** Sagittal MR image of cervicodorsal spine showing vertebral lesions with most prominent D3 vertebral lesion (arrow); **B:** Post contrast sagittal T1W SE MR image of the same patient showing lesion involving S2, S3 vertebrae (arrow) with associated large soft tissue component; **C:** T1W SE axial post contrast MR image showing right lateral epidural soft tissue enhancing component (arrow) causing cord compression.

poorly differentiated neoplasm. Serum alpha feto protein (AFP) level was more than 500 IU/mL (normal AFP level < 10 IU/mL). Ultrasound of testis, upper GI endoscopy, CT chest scan were unremarkable but CT scan of the abdomen revealed a large well defined hypodense lesion, 7.5 cm × 5.8 cm in dimensions with areas of necrosis, in the right liver lobe, in segment V, with no involvement of the portal vein. Additionally there was a destructive lesion in the right ala of the sacrum associated with a bulging soft tissue component, 5.3 cm × 3.9 cm in dimensions, which corroborated with MRI spine and bone scan findings. The patient underwent CT guided FNAC and Tru-cut biopsy of the right liver lobe lesion. Histopathological examination (HPE) was suggestive of poorly differentiated primary HCC, showing increased trabecular thickness and cellular pleomorphism with vesicular nuclei (Figure 1). Viral marker profile was positive for Hepatitis B surface antigen (HbsAg) and was non-reactive to hepatitis C virus. He was diagnosed as having primary HCC with bone metastases. The option of palliative chemotherapy was declined by the patient and he was started on palliative external beam radiotherapy (EBRT) to the right pelvis in view of pain. He developed acute onset paraplegia along with urinary retention shortly after initiation of EBRT. Immediate intravenous Solumedrol and other supportive measures were taken. Whole spine MRI was done and revealed multiple vertebral metastases at D2-D3, L1, L5, S1,S3

vertebral levels (Figure 2A and 2B) and epidural deposits with extradural cord compression at D2 and D3 vertebrae (Figure 2C). FNAC from vertebral metastases (Figure 3) revealed metastatic carcinoma consistent with primary HCC. Decompression and fixation surgery was suggested, but the family did not opt for any surgical intervention. He continued to receive EBRT for SCC. Although, there was symptomatic improvement in the pain intensity, there was no significant improvement in the neurological status. After further discussion with the family, oral chemotherapy was performed with Capecitabine and Thalidomide with no response after two cycles. Despite prompt treatment of SCC with steroids and EBRT, the patient succumbed to the disease five months after primary diagnosis.

Case 2

A 52-year-old non-alcoholic man presented with complaints of heaviness and discomfort in the right upper abdomen of two months duration. Whole abdomen ultrasound done elsewhere was suggestive of liver mass. Clinical examination revealed presence of mild hepatomegaly. Laboratory investigations showed a normal hematological profile and mild derangement of liver function tests (LFTs). Viral marker profile was positive for HbsAg and non-reactive to HCV. Whole abdomen CT scan revealed mild hepatic enlargement with an ill-defined hypodense lesion of 3.8 cm in diameter, in segment V and VI of the liver with no involvement of portal vein. His AFP levels were 3540 IU/mL. Magnetic resonance cholangio pancreatography (MRCP) revealed a nodular outline and texture of liver consistent with cirrhosis, with a focal lesion in segment V and VI, measuring 3.5 cm × 4.0 cm × 4.1 cm. He underwent CT guided FNAC from right liver lobe mass which was suggestive of primary HCC. Whole body 99 m Tc bone scan revealed no evidence of bone metastases. He was diagnosed as having primary HCC with no evidence of extra-hepatic spread. He underwent hepatic segmentectomy. HPE of the resected specimen of liver segment revealed poorly differentiated HCC with cirrhosis and regenerative nodules. CT scan of the abdomen after surgery as a part of reevaluation revealed a new focal hypodense lesion measuring 2.4 cm × 2.5 cm, in the caudate lobe of the liver and another

ill defined hypodense lesion in the right lobe of the liver with an expansile lesion with soft tissue component in the right seventh rib. Whole body 99 mTc bone scan showed irregular radiotracer uptake in the right seventh rib anterolaterally. He underwent FNAC from right seventh rib, which revealed metastatic deposit of HCC. There was a rise in serum AFP level from 3540 IU/mL at the time of diagnosis to 7920 IU/mL, suggestive of relapse. He received palliative chemotherapy with Gemcitabine and Cisplatin. CT scans after three cycles of chemotherapy revealed complete regression of caudate lobe, right liver lobe and right seventh rib lesion with a new development of a well defined lytic lesion in L2 vertebra. He also had pain in the right hip joint. Chemotherapy was continued with the same protocol for three more cycles. Subsequent CT chest scan revealed development of a new lesion in the left third rib with destruction of the left transverse process of D3 vertebra along with erosion of D5 vertebra. CT guided FNAC from the third left rib lesion was positive for malignant cells, consistent with primary in liver. He received palliative EBRT to the symptomatic areas like right hip joint, C7 to D7 vertebrae and L1 to L4 Vertebrae. One month later, he developed paraplegia with complete loss of bladder and bowel control. MRI of Dorso-lumber spine was suggestive of metastatic lesion at dorsal and lumbosacral spine with cord compression at the D6 vertebral level. The patient underwent D5 to D6 laminectomy with posterior fixation. HPE of laminectomied specimen was reported as metastatic carcinoma consistent with primary HCC. Postoperatively there was no significant improvement in the paraplegia and he did not regain bladder and bowel control. He was supported with intravenous steroids, bisphosphonates and physiotherapy. After laminectomy, although palliative EBRT was planned to the dorsal spine, (D4 to D7) vertebrae and to the lower dorsal spine, his general condition deteriorated and he succumbed to his illness 11 mo after the primary diagnosis.

Case 3

A 70-year-old man who was alcoholic, diabetic and a smoker, presented with complaints of pain in the right side of the chest. Clinical examination revealed mild hepatomegaly and pallor. Whole abdomen CT scan revealed left liver lobe mass suggestive of primary HCC with portal vein thrombosis and abdominal lymphadenopathy. He underwent MRCP and MR angiography, which revealed a soft tissue lesion in the left lobe of the liver with portal vein thrombosis and abdominal lymphadenopathy, with no signs of cirrhosis of liver. His hematological and biochemical profile were within normal range. HbsAg and HCV were not reactive in viral marker profile. His serum AFP level was 250 IU/mL. Tru-cut biopsy of left liver lobe lesion was consistent with the diagnosis of HCC. Whole body 99 mTc bone scan revealed no evidence of bone metastases at the time of diagnosis. Chemotherapy was given with Gemcitabine and Capecitabine. After the second cycle, he had sudden onset of weakness of bilateral lower limbs. Neurological examination revealed paraplegia with sensory loss below D5 level with moderate sphincteric involvement. Whole spine MRI showed extradural cord compression at D4 and

D5. Whole body 99 mTc bone scan revealed abnormal areas of radiotracer uptake in D4 and D5 vertebrae, consistent with bone metastases. Emergency D1 to D4 vertebral laminectomy was performed with posterior fixation from C7 to D5 vertebrae. HPE of laminectomied specimen showed metastasis from HCC. He received EBRT to the spine along with aggressive physiotherapy and with other supportive measures after surgery. He achieved good neurological and symptomatic recovery, and was discharged in the hemodynamically stable condition. As the patient was not willing to receive further treatment, he was followed up on palliative supportive care. However, he succumbed to the disease four months after the diagnosis.

Case 4

A 62-year-old normotensive, non diabetic, nonalcoholic man presented with complaints of pain in neck and lower back of one month duration. Pain radiated from neck to fingers bilaterally. He also had difficulty in walking due to radicular pain. Clinical examination revealed painful neck movement and mild hepatomegaly. Hematological profile was normal mild derangement of liver enzymes. Both HbsAg and HCV were non-reactive. Whole abdomen ultrasound revealed a well defined solid mass, measuring 8.4 cm × 4.9 cm, in the right lobe of the liver, with heterogeneous echotexture. Upper abdomen CT scan also revealed enlarged liver with a large hypodense lesion, measuring 10.5 cm × 10.1 cm, in the right lobe of the liver. Whole spine MRI was suggestive of multiple bone metastases in C5-C6, D9, D11-D12 and L4 vertebrae and the bodies of D9 and L4 vertebrae revealed wedge compression fracture. A small anterior epidural space soft tissue collection was also present, causing compression of the thecal sac at L4 vertebral level. Whole body 99 mTc bone scan revealed increased radiotracer uptake in D7, D9, D11-D12 and the L4 vertebrae, sacrum and bilateral sacroiliac joints, suggestive of bone metastases. He underwent CT guided FNAC from right liver lobe mass which revealed primary HCC. FNAC from vertebra was consistent with metastasis from primary HCC. Serum AFP level was elevated to 121 IU/mL at the time of diagnosis. He was diagnosed as having primary HCC with multiple bone metastases with SCC, manifesting primarily as radiculopathy. The patient and the family were given the options of surgical decompression as well as chemotherapeutic management, which they were not inclined for in view of disseminated disease. Hence, he was started on palliative EBRT to L2-L5 vertebrae and to C4-C7 vertebrae. EBRT to the thoracic spine was performed later. After completion of radiotherapy (RT) there was symptomatic improvement in the pain intensity. However, after EBRT, there was progression of SCC leading to development of paraparesis with urine incontinence. He was managed conservatively in view of deteriorating general condition. He died from his illness three months after the primary diagnosis.

DISCUSSION

HCC is the most common solid organ tumor with a high mortality^[2]. Most patients with HCC present with right upper quadrant pain or an abdominal mass due to the

presence of hepatomegaly^[7,8,9]. Rarely though, the patients may have initial symptoms related exclusively to the extra-hepatic metastases^[6]. Serum AFP level is the most useful serum tumor marker for primary HCC. Serum AFP level is elevated in most of the patients, which is highly specific for a tumor larger than three cm in diameter, and also is of prognostic value as rise after initial effective chemotherapy or surgery suggests a relapse^[3]. This was evident from the fact that AFP level was elevated in all our cases and relapse occurred after hepatic segmentectomy in the second patient corresponded to the elevation of AFP levels.

Extra-hepatic spread from HCC is not uncommon and reported to be 30%-78% at autopsy examination. Bone metastases are rare with an incidence of only about 2%-20%^[2-5,10,11]. Although incidence of metastasis of HCC to the bones is low, recent reports have shown an increasing incidence and is estimated to be about 28%^[1].

The most frequent sites of the bone metastases are ribs, spine, femur, pelvis and humerus according to Khulman *et al*^[2]. Patients with bone metastases most often present with pain as the principal symptom^[3,7]. Two of our patients presented with the chief complaints of backache. Very rarely patient may present with bone pains without any symptom of underlying hepatic pathology, as seen in our first case.

In the majority of cases, vertebral body metastases result from hematogenous dissemination of tumor which is evident by the vertebral column bone marrow involvement^[6,10]. Radiologically, bone metastases from HCC appear osteolytic on plain films. All the four patients in our series had osteolytic lesions. They are destructive, expansive and often associated with large soft tissue masses^[2-6,10]. Conventional radiography is however, not a very sensitive modality for the diagnosis of early bone metastases as the cancellous part of the bone is usually the first site of bone metastases and cortical part of the bone is responsible for most of the bone density depicted on plain X-Ray films^[6]. For the same reason the bone scan is also less sensitive. MRI is most helpful for early diagnosis as well as delineation of the extent of metastases^[2,6].

The histological appearance of the bone metastases from primary HCC is similar to that of the primary tumor, with positive bile staining^[2,4]. Recent insight into the causation of bone metastases of HCC has been correlated with angiogenesis^[8]. Significant hemorrhage from metastatic lesions is reported to occur either spontaneously or after biopsy of the lesion^[2].

The level of vascular endothelial growth factor (VEGF) has been reported to be elevated in HCC with bone metastases^[8]. VEGF, the most important angiogenic factor, has been shown to stimulate bone resorption through its effects on osteoclasts^[8,12]. Thus in the era of targeted therapy, VEGF could be an important target for the treatment of these tumors. In their study on metastatic HCC, S. Kummar *et al* have evaluated TNP-470, a derivative of fumagillin and a potent angiogenetic inhibitor, as a treatment for experimentally induced HCC in animal models^[8]. Similarly, the serum levels of C-terminal telopeptide of type 1 collagen are also significantly elevated in patients with bone metastases^[13].

The overall frequency of the malignant SCC has been

reported to be approximately 5% in cancer patients. In approximately 95% of cases, SCC is caused by extradural metastases from tumors involving the vertebral column. Thoracic spine (70%) is most commonly involved as compared to the lumbosacral (20%) and the cervical spine (10%)^[12,14]. Three cases reported by us had SCC at thoracic vertebrae level and all of them had extradural compression. Although SCC is rare with HCC, the symptoms and involvement are similar to other primary tumors, commonly leading to SCC, such as lung, breast and prostate cancer.

Omura *et al* in 1989 described a case of a 57-year-old male with primary HCC diagnosed when he developed paraplegia secondary to a SCC due to vertebral tumor. After laminectomy, the tumor histology was reported to be metastatic HCC^[15]. Kantharia *et al* in 1993, also described a case of radiculopathy and rapidly developing SCC, due to bone metastases and diagnosed as HCC at autopsy^[6]. Pinazo Seron *et al* in 1999 reported a case of a 55-year-old man, who had alcoholic cirrhosis and HCC. This patient developed SCC due to soft tissue epidural metastases, seated at the paravertebral zone. Plain radiography and bone scan were normal and diagnosis was achieved by MRI and FNAC^[16]. Cho *et al* in 2002 reported a case of pathologically confirmed HCC who developed lower leg weakness, which was found to be due to spinal metastases as evidenced by MRI spine. This patient received emergency radiotherapy (RT) and recovered from SCC^[17]. Melicher *et al* in 2002 also described a case of asymptomatic liver mass of uncertain histology of one year duration. He presented with back pain and developed signs of SCC, and he underwent laminectomy, which established the diagnosis of metastatic HCC^[18].

Cord compression occurs due to invasion of epidural space, most often as a direct extension of vertebral body metastases. There are various routes of the epidural invasion by tumor cells, hematogenous being the most common mode of spread. Hematogenous spread occurs directly or *via* the involvement of Batsons venous plexuses^[19].

In metastatic SCC, back pain is not only the most common symptom but also the earliest manifestation of SCC, as seen in two of our patients. First sign after development of pain is weakness due to myelopathy. Once lost, neurological functions cannot be regained in most of the patients^[12,20,21].

Though various modalities such as plain radiography, CT scan, CT-myelography and MRI can be used to evaluate SCC, whole spine MRI is the best method of evaluating epidural SCC. Apart from being most sensitive, cost-effective and noninvasive, MRI is also helpful in distinguishing between benign and malignant causes of SCC^[20,21]. In all our patients, SCC was diagnosed with the help of MRI spine, which revealed the site of extradural cord compression with precision. Presently CT-myelography is used only for patients in whom MRI is contraindicated^[21].

The pre-treatment degree of neurologic dysfunction is the strongest predictor of therapeutic outcome. However, the most important weapon against the prevention of devastating complications of SCC is the high index

of suspicion and the awareness that SCC is a potential oncologic emergency^[20]. Thus the development of any new pain or any change in the character of the pain mandates complete neurological examination along with MRI whole spine as a screening modality to rule out early signs of SCC^[21].

The treatment modalities available for SCC are individualized with a definite role of corticosteroids, RT, chemotherapy and surgery. Corticosteroids act by relieving edema and help preserve neurological function. They may also improve overall outcome after specific therapy^[19,22,23].

RT is an important part of the management of SCC and it helps in pain relief, cytoreduction of tumor, prevention of progressive neurologic dysfunction and structural damage to the cord. RT reduces pain in approximately 70%, improves motor function in 45% to 60%, and reverses paraplegia in 11% to 21% of the patients. The outcome of RT is related to the neurological status prior to the treatment and the radiosensitivity of the tumor. RT should also be given following surgery in patients who have not previously received radiation^[24-27]. Our third patient was given EBRT to the affected region after emergency posterior laminectomy was performed.

Surgical interventions are usually indicated in situations where the diagnosis is unknown, in cases of spinal instability or compression by the bone requiring prompt relief of pressure related symptoms^[12,20,27]. These four cases suggest that surgery should be the integral part of SCC management in HCC. Our first patient immediately received RT after developing SCC, with no improvement in neurological status, and in the second patient, SCC developed at the vertebra which was earlier irradiated. RT did not help prevent paraparesis in the fourth patient. Whereas, the third patient who underwent emergency laminectomy followed by EBRT had best palliation. Surgery should also be considered in the patients who fail to respond to RT or deteriorate further while on RT and the patients who have received maximal allowable radiation dose to the spinal cord^[12,20]. The surgical approach should be determined based on the location of vertebral involvement and the direction of compression^[20]. The two main surgical approaches used for decompression are laminectomy with posterior fixation and anterior decompression of the spine with reconstruction. Anterior decompression of the spine with mechanical stabilization has been accepted as the surgical intervention of choice for anterior vertebral body involvement^[21,28]. Our second and third patient underwent posterior laminectomy for emergency decompression without any immediate morbidities. But the aim is to diagnose SCC in the early stage so that emergency decompressive surgery will not be needed.

CONCLUSION

Extra-hepatic spread of HCC is usually to the lungs and regional lymph nodes, but rarely to the bones. SCC due to vertebral bone metastases is a rarer phenomenon. SCC is an oncologic emergency and treatment delay can lead to irreversible consequences. These four cases highlight unusual metastatic presentation of HCC, in the form of

bone metastases resulting in SCC. High index of suspicion is required to achieve good outcome in patients who are at risk to develop SCC secondary to bone metastases. Thus, any change in the character of already existing bone pain or new development of pain mandates a complete neurological examination along with MRI of the whole spine to rule out cord compression. Although, radiation, steroids and surgery can be used as treatment modalities, surgery followed by EBRT can result in better palliation. But the aim is to diagnose SCC at an early stage so as to prevent the devastating consequences of this complication.

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Fulminant hepatic failure caused by *Salmonella paratyphi A* infection

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Abstract

We report a case of fulminant hepatic failure associated with *Salmonella paratyphi A* infection, in a 29-year-old patient who was admitted to the intensive care unit (ICU) with fever of two days, headache and vomiting followed by behavioural changes and disorientation. On examination, the patient appeared acutely ill, agitated, confused, and deeply jaundiced. Temperature 38.5°C, pulse 92/min, blood pressure 130/89 mmHg. Both samples of blood grew *S. paratyphi A*, which was sensitive to ceftriaxone and ciprofloxacin. Ceftriaxon was administered with high-dose dexamethasone. Two weeks after treatment with ceftriaxon, the patient was discharged in satisfactory condition.

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Key words: Typhoid hepatitis; Fulminant hepatic failure; *Salmonella paratyphi A*

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INTRODUCTION

Fulminant hepatic failure is a dramatic clinical syndrome characterized by massive necrosis of liver cells^[1]. It is caused by acute viral hepatitis A, B, C, D, E (40%), other viruses, drugs, Weil's disease, Wilson's disease, acute fatty liver in pregnancy, ischemic hepatitis, acute Budd-Chiari, malignant infiltration or bacterial infection including *salmonella*.

Although salmonella hepatitis is a rare presentation of typhoid fever, fulminant hepatic failure is extremely uncommon, few cases were reported with such presentation.

To our knowledge, this is the first reported case of fulminant hepatic failure in the state of Qatar, associated with *Salmonella paratyphi A* infection.

CASE REPORT

A 29-year-old Nepali male patient admitted to the intensive care unit (ICU) through accident and emergency (A&E) department with fever for two days, headache and vomiting followed by behavioral changes and disorientation. The patient returned back from his country two months ago. No other history of possible relevance was found. On examination, the patient appeared acutely ill, agitated, confused, and deeply jaundiced. Temperature was 38.5°C, pulse 92/min, and blood pressure 130/89 mmHg. Examination of the nervous system showed a disoriented patient, moving all his limbs, with incoherent speech and negative meningeal signs. Examination of the abdomen revealed mild splenomegaly. Chest and heart were unremarkable. Hemoglobin level was 150 g/L, total leucocyte count 5000/ μ L (60% neutrophils, 31% lymphocytes) and adequate number of platelets. Blood chemistry showed aspartate aminotransferase (AST) level of 966 IU/L, alanine amino-transferase (ALT) 176 IU/L, alkaline phosphatase 267 IU/L, total bilirubin 180 μ mol/L, direct bilirubin 109 μ mol/L, total proteins 74 g/L, albumin 38 g/L, urea nitrogen 3 mmol/L, creatinine 44 μ mol/L, sodium 135 mEq/L and potassium 3.6 mEq/L, bicarbonate 22 mmol/L, Ca 2.2 mmol/L, ammonia level 100 μ mol/L (normal up to 50 μ mol/L), prothrombin time 30.8 s and an international normalized ratio (INR) of 3.1. Macroscopic and microscopic examination of urine and stool were normal. Viral markers for hepatitis A, B, C, Monospot test and cytomegalovirus serology were negative. Antismooth muscle antibody and antimithochondrial antibody were negative. Peripheral smear for malaria was negative on several occasions. Lumber puncture was done, and CSF study was normal.

Computerized tomography (CT) scan of the head with intravenous contrast was normal. Electroencephalography showed findings suggestive of metabolic encephalopathy. Chest X ray was normal. Ultrasonography of abdomen revealed enlarged spleen. Rest of abdomen including liver, gall bladder, and kidney were all within normal limits.

A CT scan of the abdomen, with intravenous contrast showed moderate spleen enlargement and liver enlargement with homogeneous parenchyma. There was no evidence of abscess or tumor. No evidence of mass or fluid collection was seen in the abdomen or pelvis. Kidneys and

Table 1 Laboratory data on admission and discharge

	Alanine amino- transferase (ALT)	Aspartate aminotrans- ferase (AST)	Alkaline phosphatase (ALP)	Total bilirubin
On admission day	176 IU/L	966 IU/L	267 IU/L	180 µmol/L
On discharge day	65 IU/L	105 IU/L	110 IU/L	33 µmol/L

pancreas were within normal limits. Echocardiography was normal. Magnetic resonance image (MRI) of the abdomen with MR cholangiography showed peri-portal edema within the liver and collapsed gall bladder, suggesting the possibility of hepatitis.

The patient was admitted to the ICU as a case of fulminant hepatic failure evidenced by marked elevation of hepatocellular enzymes, prolonged prothrombin time, hyperbilirubinemia and hyperammonaemia. The patient was given vitamin K and fresh frozen plasma to correct the prolonged INR, and Lactulose (enema and oral). Broad-spectrum antibiotic and high dose dexamethasone (3 mg/kg loading dose over 30 min, followed by 1 mg/kg every six hours for two days) were initiated. Cultures of urine were sterile and no pathogen was isolated from stool culture.

However, both samples of blood grew *S. paratyphi A*, which was sensitive to ciprofloxacin and ceftriaxone. Once the culture report was available the treatment was changed to ceftriaxone 2 gm twice daily. After one week of therapy, the patient was afebrile and oriented, and the dose of ceftriaxone was reduced to 2 gm daily for one more week. Two weeks after treatment with ceftriaxone, the liver enzymes dropped obviously (Table 1). Consequently, the patient was discharged in satisfactory condition.

DISCUSSION

Typhoid fever may be an important cause of illness and death for centuries, although historical accounts do not clearly distinguish it from other febrile illnesses. It has been implicated in the death of Alexander the Great, in 323 B.C.^[2]

The causative organism widely spreads in all parts of the world, although the disease is more prevalent in developing countries than in developed ones. The most common cause of typhoid fever is *Salmonella enterica* serotype typhi, although salmonella of other serotypes, particularly *Salmonella enterica* serotype paratyphi A, can cause a similar enteric fever. *Salmonella enterica* serotype typhi has no animal reservoir.

In typhoid, involvement of liver is a consistent feature^[3,4]. Typhoid hepatitis is a rare presentation of typhoid fever, clinically suspected in patients with persistent fever, hepatomegaly and jaundice and especially in cases whose liver function tests show predominantly conjugated hyperbilirubinemia, modest elevation of liver enzymes and negative serology for viral hepatitis^[5,6].

The differential diagnosis of fever in the icteric phase of hepatitis, include: viral hepatitis, malaria hepatitis, Weil's disease, typhoid hepatitis, autoimmune hepatitis and drug

induced hepatitis.

In this patient, viral markers for hepatitis A, B, C, Monospot test and cytomegalovirus serology were negative. Peripheral smear for malaria was negative on several occasions. Antismooth muscle antibodies (Ab) and antimitochondrial Ab were negative and whether the patient had taken any hepatotoxic drugs was unknown.

The common complications of typhoid fever include relapse, perforation and hemorrhage from bowel ulcerations^[3]. Extreme hepatic dysfunction with hepatic encephalopathy is a rare coexisting complication in salmonella hepatitis.

The pathogenesis of severe hepatic involvement in salmonella infection may be multifactorial, involving endotoxin, local inflammatory and/or host immune reactions.

In typhoid hepatitis, liver biopsy would have shown pathognomonic lobular aggregates of Kupffer's cells-so-called typhoid nodules, but liver biopsy was not performed in this case^[2].

Although, viral markers for hepatitis E and leptospiral test were not made in this case, the diagnosis of fulminant hepatic failure due to *S. paratyphi A* was based mainly on three facts: first, positive blood culture for *S. paratyphi A*; second, elevated alkaline phosphatase level, aspartate aminotransferase (AST) level higher than alanine aminotransferase (ALT), and only mild prolongation of the prothrombin time^[7]; and third, excellent response to ceftriaxone and dexamethasone^[7].

The most important aspect of treatment for typhoid fulminant hepatic failure is to provide good intensive care support, recognize the condition promptly and initiate early dexamethasone and proper antibiotics. Monitoring the complications and instituting appropriate therapy are also critical.

The current drug of choice for adults is ciprofloxacin, which combines little documented resistance with excellent penetration into macrophages and the biliary system. This may lower the rate of relapse and chronic carrier states. Alternative antibiotics can be used if sensitivities are known or suspected resistance is low^[8]. The current drug of choice for children and pregnant women is parenteral ceftriaxone^[9].

Thus, in patients from endemic areas, typhoid hepatitis should be considered in the differential diagnosis of fulminant hepatic failure since early institution of specific therapy in these cases yields a good prognosis.

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CASE REPORT

Multi-limb necrotizing fasciitis in a patient with rectal cancer

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Abstract

Necrotizing fasciitis is a devastating soft tissue infection affecting fascias and subcutaneous soft tissues. Literature reviews have identified several related risk factors, including malignancy, alcoholism, malnutrition, diabetes, male gender and old age. There are only scanty case reports in the literature describing its rare association with colorectal malignancy. All published cases are attributed to bowel perforation resulting in necrotizing fasciitis over the perineal region. Isolated upper or lower limb diseases are rarely identified. Simultaneous upper and lower limb infection in colorectal cancer patients has never been described in the literature. We report an unusual case of multi-limb necrotizing fasciitis in a patient with underlying non-perforated rectal carcinoma.

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Key words: Necrotizing fasciitis; Soft tissue infection; Colorectal cancer; Malignancy; Group G streptococcus

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INTRODUCTION

Necrotizing fasciitis is a devastating soft tissue infection affecting fascias and subcutaneous soft tissues^[1]. Two distinctive clinical entities are recognized: hyperacute and subacute variants^[2]. Hyperacute necrotizing fasciitis usually manifests as a rapidly progressing infection with resultant multi-organ failure due to group A streptococcus or clostridium species^[3]. Contrarily, polymicrobial infection is implicated in subacute necrotizing fasciitis that represents a slowly evolving disease with less fulminant systemic complications^[4]. Regarding its etiology, necrotizing soft tissue infection can be secondary to trauma, wound

infections, perianal or urogenital abscesses, decubitus ulcers or idiopathic in origin^[5]. Malignancy, alcoholism, malnutrition, diabetes, male gender and old age are general risk factors associated with necrotizing fasciitis^[6,7]. There are only scanty case reports in the literature describing its rare association with colorectal malignancy^[8-13]. All these cases are attributed to bowel perforation resulting in necrotizing fasciitis over the perineal or abdominal region. Isolated upper or lower limb diseases are exceedingly uncommon. Multi-limb necrotizing fasciitis is even rarer. We report here the first case of multi-limb involvement of necrotizing fasciitis in a patient with underlying rectal carcinoma.

CASE REPORT

A 56-year-old male patient, who had a medical history of alcoholic cirrhosis, poorly-controlled diabetes mellitus and mild renal impairment, presented with an one-month history of per rectal bleeding. Digital rectal examination revealed a non-obstructing rectal tumour situated at 8 cm above the anal verge. Tumour biopsy was taken through proctoscope and the histology confirmed it to be an adenocarcinoma. No abdominal discomfort or increase in per rectal bleeding was reported after the procedure. However, one day after the tumour biopsy, he complained of bilateral lower limb pain over the pre-existing ankle edema. He denied any previous history of trauma. No wound or skin abrasions could be identified over both lower limbs. In addition to the swollen lower limbs, he was noticed to have unexplained sinus tachycardia (pulse rate 100 per min) despite normal haemodynamic status and oxygen saturation. Examination of other organ systems was unremarkable. The arterial blood gas, white cell counts, chest radiograph and electrocardiogram were normal. Though Doppler ultrasound did not show any evidence of deep vein thrombosis in both lower limbs, low molecular weight heparin was commenced as pulmonary embolism was not excluded.

His clinical condition rapidly deteriorated over the next 24 h when he developed acute on chronic renal impairment, hypotension and worsening metabolic acidosis that necessitated inotropes and hemodialysis support in the intensive care unit. Urgent ultrasound of the abdomen did not reveal any evidence of obstructive uropathy. He became hypothermic (core temperature 32°C), drowsy and mentally confused. Blood parameters revealed marked leukocytosis and disseminated intravascular coagulation. Empirical intravenous antibiotics including augmentin (amoxycillin and clavulanate), clindamycin and metronidazole were commenced in view of the clinical

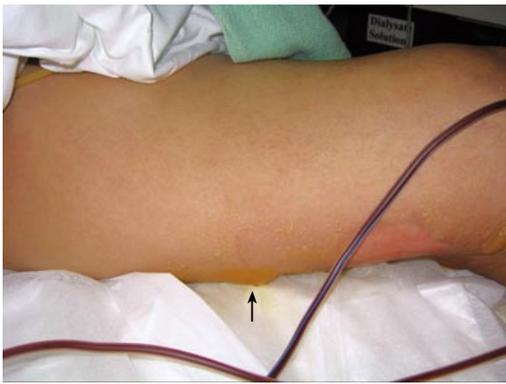


Figure 1 Enlarging blisters over the thigh. One of the blisters is indicated by black arrow.



Figure 2 Necrotic skin patches over dorsum of hand.

Table 1 Literature review of necrotizing fasciitis associated with colorectal malignancy

Authors	Yr published	Patient sex	Colorectal cancer type	Predisposing factors	Location of necrotizing fasciitis	Bacteria cultured	Outcome
Dewire <i>et al</i> ^[8]	1992	Male	Sigmoid	Bowel perforation	Fournier's gangrene	<i>E coli</i> , <i>Bacteroides fragilis</i> , enterococci, anaerobes	Alive
Lam <i>et al</i> ^[9]	1996	Male	Sigmoid	Bowel perforation	Psoas abscess to upper thigh	<i>E coli</i> , <i>Bacteroides fragilis</i> , <i>Edwardsiella tarda</i> , staphylococcus, & Group G streptococci	Death
Gould <i>et al</i> ^[10]	1997	Male	Sigmoid	Bowel perforation	Fournier's gangrene	Group F streptococci, anaerobes	Death
Lawrentschuk <i>et al</i> ^[11]	2003	Male	Rectum	Perforation with abscess	Fournier's gangrene	<i>E coli</i> , Enterococcus, anaerobes & staphylococcus epidermidis	Alive
Gamagami <i>et al</i> ^[12]	1998	Male	Rectum	Perforation with abscess, Diabetes	Fournier's gangrene	<i>E coli</i> , enterococci	Alive
Ku <i>et al</i> ^[13]	2006	Female	Transverse	Perforation with invasion to rectus fascia	Abdominal wall	<i>Klebsiella pneumonia</i>	Alive

features of sepsis. Shortly after his stay in the intensive care unit, blisters (Figure 1) were discovered on both swollen lower limbs. Necrotizing fasciitis was suspected and ultimately confirmed with surgical exploration 12 h after the appearance of blisters. Initially, fasciotomy and excisional debridement were endeavored in an attempt to preserve the lower limbs. Meanwhile, high dose penicillin was introduced to replace augmentin. Nevertheless, the worsening clinical sepsis and migratory soft tissue necrosis inevitably resulted in bilateral above knee amputation. Microbiological cultures of blood and necrotic tissues invariably grew group G streptococcus only. Histological examination of the excised tissue also confirmed the diagnosis of necrotizing fasciitis. One day afterwards, similar features of skin changes developed over both upper limbs (Figure 2) that required repeated excisional debridements. In spite of limb amputation, surgical debridement and parenteral antibiotics, he developed fulminant group G streptococcal septicaemia and deteriorated rapidly until he succumbed to multi-organ failure six days after the onset of sepsis. Throughout his disease course, the perineum, groin and abdominal wall remained uninvolved.

DISCUSSION

In the English literature, there are only six reported cases of necrotizing fasciitis associated with colorectal malignancy (Table 1). In these cases, necrotizing fasciitis was all preceded by perforation of colorectal tumours. All except two cases^[8,10-12] presented as Fournier's gangrene.

One case manifested as psoas abscess with spreading infection to the thigh after retroperitoneal perforation of a sigmoid tumour^[9]. The other one involved the anterior abdominal wall as a result of direct tumour invasion and perforation from the transverse colon^[13]. In essence, perforated colorectal tumours represent an entry focus for bacterial translocation that might penetrate the subcutaneous soft tissues, leading to necrotizing infection^[14]. To our knowledge, our patient is the first reported case of disseminated necrotizing fasciitis affecting all limbs in a non-perforated rectal tumour.

With regard to its etiology, we postulate three causes for such a fulminating disease in our patient. First, chronic subcutaneous tissue edema due to cirrhotic hypoalbuminaemia predispose him to subcutaneous tissue infection^[6]. In addition, systemic immunosuppression could well be secondary to the cumulative effects of malignancy, diabetes mellitus and cirrhosis^[7]. Lastly, transient bacteraemia might have arisen as a consequence of bacterial translocation emanated from occult necrosis of his rectal tumor^[14]. Interestingly, there was a close temporal association between tumour biopsy and the onset of necrotizing sepsis in our patient. Transient bacteraemia following endoscopic tissue biopsy has been reported^[15]. However, Gram negative organisms were the main bacteria retrieved and it was elusive to verify the causative association between rectal biopsy and necrotizing soft tissue infection in our patient.

So far as the bacteriological origin was concerned, group G streptococcus was the only culprit in our patient. In 1935, Lancefield, an American microbiologist, first

described a Gram positive facultative anaerobic coccus as group G streptococcus that constitutes parts of the normal flora of human skin, respiratory tract and gastrointestinal tract^[16-18]. Although it has been rarely implicated in necrotizing fasciitis^[19], its associated mortality could be comparable to those induced by streptococcus pyogenes^[20], suggesting that group G streptococcus, being a normal flora of the gastrointestinal tract, could represent a potentially life-threatening pathogen for all patients^[20].

Regardless of the disparity in pathological and microbiological etiologies, the mortality rate of necrotizing fasciitis remains high. High index of suspicion and prompt surgical intervention are the cornerstone of treatment for improving the disease outcome^[7]. We believe that delay in diagnosis and surgical treatment contributes to the demise of our patient.

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Cardiac mucosa indicates risk for Barrett esophagus

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TO THE EDITOR

With interest we read the article by Bani-Hani *et al*^[1] entitled "Pathogenesis of columnar-lined esophagus", which has been published in the recent issue of *World Journal of Gastroenterology*. The review profoundly adds to our understanding of columnar-lined esophagus (CLE) and clearly indicates that CLE represents an acquired condition and develops as a consequence of gastroesophageal reflux disease (GERD)^[1]. However, it should be pointed out that inclusion of CLE-histopathology helps to define those at risk for dysplastic and malignant transformation. Histopathology characterizes *nondysplastic* and *dysplastic* (low-, high grade dysplasia) CLE^[2-4]. Going in line with the *Paull- Chandrasoma* classification^[2], *nondysplastic* CLE includes oxyntocardiac mucosa, cardiac mucosa, multilayered epithelium (a mixture of basal layer of squamous epithelium and cardiac mucosa) and cardiac mucosa with goblet cells (i.e.

intestinal metaplasia; *Barrett* esophagus, BE). In contrast to cardiac mucosa, goblet cells have never been detected within oxyntocardiac mucosa^[2,3]. Consequently, following a 4-quadrant biopsy protocol of the esophagogastric junction (including squamous and gastric type mucosa, irrespective of the presence or absence of endoscopic CLE) presence of oxyntocardiac mucosa in all biopsies indicates absence of risk to progress towards intestinal metaplasia and dysplasia^[2,3]. In contrast to that, presence of cardiac mucosa indicates risk to undergo intestinal metaplasia and progress towards dysplasia and adenocarcinoma of the esophagus (annual incidence for BE 0.2%-2.0%). Controversy still exists if CLE is esophageal or gastric. Normally the esophagus is lined by squamous epithelium and the stomach is covered by oxyntic mucosa. Recently Chandrasoma *et al*^[4] showed that CLE, but not oxyntic mucosa, is present above submucosal glands and adjacent to submucosal gland ducts. Since submucosal glands are absent in the stomach, CLE is considered to be esophageal, irrespective of endoscopic appearance^[2-4]. Taken together, CLE represents the morphologic consequence of GERD. GERD should be included into histopathologic routine. Hopefully, future studies will evaluate the incidence and prevalence of CLE subtypes in the normal population for identification of those at risk for intestinal metaplasia and adenocarcinoma of the esophagus.

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- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci U S A* 2006; In press

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- 5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764]

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- 12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wiczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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- 13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ Cell Tumour Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/EID/eid.htm>

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